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Utilization of Palm Oil Mill Effluent for Bio-Extract Production: Physical, Chemical and Microbial Properties, and Application to Growth of Chinese Kale

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ABSTRACT

Palm oil mill effluent is composed of several organic compounds such as nitrogen compounds, oligosaccharides, organic acids, and lipids, which may be beneficial when applied to bio-extracts. The aim of this study was to determine the miscibility of palm waste as an ingredient for bio-extract production; the waste liquid was mixed with two other agricultural wastes: fish waste and pineapple peel. Physical, chemical, and biological changes such as phytotoxicity of the product were observed. Bio-extract fermentation was complete around the end of the first month. The completion of fermentation was determined by the physical, chemical and microbiological features of the mixture. The physical changes of the bio-extract were not significantly different after 35 days of fermentation. Reducing sugar content declined from 66.3 to 2.3 g L^{-1} over the 150 days of fermentation. COD decreased as well, from 5300 (after secondary treatment) to 200 mg L^{-1}. This could be due to the effect of fermentation on hydrocarbon-based compounds, which are broken down into substances that the microbes are able to digest more efficiently. This bio-extract was subsequently applied to Chinese kale at various dilutions; a dilution ratio of 1:100 was the most effective for growing Chinese kale. These results indicate that the addition of waste liquids from palm oil factories into bio-extract fermentations could be practical, especially when applied together with fish waste and pineapple peel. The reuse of waste water in bio-extracts could reduce negative impacts on the environment. Moreover, using bio-extracts has the advantage of being lower cost and more environmentally friendly than chemical fertilizers.

Keywords: Bio-extract; Chinese kale growth; Fish waste; Pineapple peel; POME

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1. Introduction

Palm oil mill effluent (POME) is a liquid waste produced during the palm oil extraction process. POME has high organic matter content and is considered one of the biggest wastewater pollutants in the world. In parallel with each ton of palm oil, approximately 5.5-7.5 tons of POME are produced [1]. Worldwide, more than 50 million m^3 of POME are produced each year [2]. The physicochemical characteristics of POME may vary according to locale and specific process characteristics (climate, species, pretreatment and oil extraction process) [3].

The nutrients from this oily substance could potentially harbor several micro-organisms, as it has a very high level of suspended organic components such as nitrogenous compounds, simple sugars, and free organic acids [4]. POME is known for its high nutritive content [5]; it contains 0.72-0.95% N, 0.12-0.18% P, and 1.90-2.30% K, as well as 288 mg/L Mg, 286 mg/L Ca, 66 mg/L Fe, 1.98 mg/L Zn, 0.85 mg/L Cu and 2.80 mg/L Mn [3]. This suggests that raw POME could potentially be made into organic fertilizers, as it is derived from naturally occurring substances and is produced via natural biological processes.

Therefore, this wastewater could be used as a nutrient source in bio-extracts. A bio-extract is generally made of anaerobically fermented plant or animal waste, supplemented with a certain source of carbon [6]. In addition to this, bio-extracts supply several organic acids and amino acids as well as several growth regulators depending on the type of ingredients included [7]. Reuse of waste water in bio-extracts could reduce negative impacts on the environment by reducing the amount of wastewater released into the environment, which is usually dumped into natural bodies of water. Recent research studies have mentioned that bio-extracts provide chemicals promoting plant growth and productivity. It has also been found to be effective when used with regular fertilizers, improving performance of agricultural products [8-10].

Fermented bio-extract, considered a local wisdom, is a mixture of several organic residues together with molasses, a dark brown residue waste product from sugar processing. In this study, pineapple peel was used in place of molasses, due to it containing several sugars and being of lower cost. Its fermentation process is both aerobic and semi-anaerobic depending on the metabolite contents. The advantage of using fermented bio-extracts is the reduced need for various other chemicals used to promote plant growth and control pests. These chemicals are considered harmful to the environment, farmers, and consumers [11]. A fermented bio-extract could improve crop quality and increase crop yield [12]. The application of bio-extract significantly improved tomato yield, greatly increasing dry weight and fruit number ($p < 0.01$). A study by Kamla et al. [13] indicated that using a combination of bio-extract and organic fertilizers resulted in higher crop biomass and yield compared to plants for which only organic fertilizers were used. In a pot experiment by local farmers, the addition of bio-extract without fertilizer did not increase either biomass or yield in cowpea plants. However, when both bio-extract and organic fertilizers were used together, they significantly increased plant dry weight and yield, more so than a combination of organic fertilizer and bio-extract. Moreover, bio-extract made from animal waste resulted in larger and more consistent yields than bio-extracts made from plant waste.

Traditionally, Thai farmers apply bio-extract to improve the productivity of both the reproductive and vegetative parts of several crop plants, including Chinese Kale, Chinese green mustard, French marigold and soybean [14]; [8-9]. It has also been proven that bio-extracts promote the growth and reproduction of several microbes such as *Bacillus*, lactic acid bacteria, and yeast which lend further benefits to plants [6]. Furthermore, Thai farmers typically consider

bio-extracts to be a vital tool for agriculture, due to its ability to reduce the need for chemical fertilizers and insecticides. Several Thai government agencies promote utilizing organic products in agriculture and encouraged local farmers to produce and use their own bio-extracts. However, the properties of the bio-extracts produced by local people significantly varies depending on the source of raw materials and additives. It' s rather difficult to produce bio- extract that is consistent in its properties and makeup, since information on bio-extract nature and qualities is not yet available, especially to local farmers. The application of a low-quality bio-extract might have adverse effects on both plant growth and productivity. In previous studies, the quantification of nutrients, plant hormones, organic acids, and total bacterial count were the sole focus. These studies found that bio-extracts produced using raw materials from different origins provided different fermentative results [7]. Factors that alter bio-extract qualities have been investigated to a certain degree, although findings thus far remain inconsistent [7]; [15]. However, the impact of microbes on the physical and chemical qualities of a bio-extract, as well as the cell count during fermentation, have been proposed in several studies. Furthermore, a number of studies have focused on the effects of bio- extracts on plant growth and productivity [8-9]. Several insights gained by these studies were mostly conducted on Chinese kale (*Brassica oleracea* var *alboglabra*), an agricultural crop regularly used in household kitchens. The aims of this study are to evaluate the miscibility of waste liquid produced from palm oil production, combined with certain other agricultural waste products generally accessible in Thailand, such as fish waste and fruit peels, to produce a bio- extract, then to evaluate the physical, chemical, and microbiological properties of the resulting bio- extract, and finally, to observe the effects of using said bio-extract on the growth and development of Chinese kale.

2. Materials and Methods

2.1 Raw materials

Fish waste from the fishing sector Nakonsrithammarat, and Trang province, and pineapple peel from a local market, both with a particle size of 10-35 mm were used as primary solid waste, together with palm oil mill effluent (POME) collected from secondary treatment effluent from a stabilization pond of Srijarean Palm Oil Co. Ltd. Krabi, Southern Thailand. First, POME was mixed with pure water at a ratio of 1: 1 v/v and left in open air for 24 h to allow for natural fermentation [10]; [16]. The physical, chemical, and biological properties of each material were then analyzed (Table 1).

2.2 Experimental

2.2.1 Bio-extract preparation

Bio- extract was prepared by thoroughly mixing fish waste, pineapple peel, and POME at a ratio of 3: 1: 1 (by weight), the mixture was subsequently held in a tightly closed vessel and kept at room temperature for 5 months. During the first 5 weeks of incubation, the mixture was analyzed every week. The process of analysis was performed as follows: a sample was taken from the vessel and centrifuged at 10,000 rpm for 5 min, the pellet was discarded and the liquid phase was kept and analyzed promptly. Physical characteristics of the liquid phase, i.e., color and texture, were observed by unaided-eye. The pH and electrical conductivity (EC) were analyzed using methods from Loh et al. [4]. The chemical oxygen demand (COD) was analyzed using the close reflux and titrimetric method, used by Ahmed et al. [5]. The total carbon content (TC) was measured using a method modified from Loh et al. [4]. Total nitrogen (TN) and reducing sugar content were determined using a method modified from Soh et al. [17].

Table 1. Characteristics of three ingredients used in bio-extract preparation.

Chemical characteristics	Ingredients		
	POME	Fish waste	Pineapple waste
pH	4.15±0.25	5.24±0.02	3.47±0.12
Total solids (%)	6.25±0.35	42.21±1.23	19.45±0.22
Oil and Grease (%)	4.32±0.31	nd	nd
COD (mg L^{-1})	5340±0.20	nd	nd
TN (% g/gDW)	0.53±0.50	14.25±0.95	0.95±0.21
TC (% g/gDW)	8.26±1.52	45.18±0.99	53.84±0.52
C/N ratio	15.60±2.48	3.20±0.89	56.70±1.84
Total bacterial (cfu/ml)	$1.15x10^5$	nd	nd
Total LAB (cfu/ml)	$4.23x10^3$	nd	nd

Nd = no data

Fermentation byproducts such as ethanol and various organic acids such as acetic acid, propionic acid, butyric acid, and lactic acid were assayed using the FID gas chromatography method, similar to the methods used by Tripetchkul et al. [10]. The amount of microbes in the bio-extract was determined by the colony count plate method; typical nutrient agar (NA) with De Man, Rogosa, and Sharpe agar (MRS) were used for determining total colony count and lactic acid bacteria count, respectively [10].

2.2.2 Growth medium preparation

Growth medium was prepared by mixing sand and coconut coir together. Sand was sieved to select for a particle diameter between 0.2 and 2.0 mm, following a method modified from Soh et al. [17]. Coconut coir was prepared by sieving coconut husk to a size of 4.0 mm or less. Sand and coconut coir were then thoroughly mixed at a ratio of 1:2 by volume.

Complete nutrient solution, used as a positive control, was prepared following a method modified from Pathanapibul [18], which is suggested to provide the appropriate solution volume adequate on daily watering. The fresh nutrient solution was added to the growth medium to compensate for plant nutrient uptake. The volume of nutrient solution together with the addition of bio-extract was considered as well. The method mentioned above required two steps of determination: first, a standard curve of water running through the pots was calculated to determine the average runout rate; second, the average runout rate was used to determine the requirement of growth media and the rate of media added to keep nutrient levels in the media and bio-extract remaining constant. In this study, each pot was given 1,100 g of bio-extract and 134 g of complete nutrient solution. Drainage volume of media and bio-extract was recorded and medium was supplied daily according to the runout rate to allow 100% medium saturation throughout the 150 days of the experiment.

2.2.3 Chinese kale seedling and nutrient solution preparation

A culture tray with 108 holes was prepared by sowing three seeds of Chinese kale into each hole. The holes were initially watered with distillated water until a shoot sprout had emerged for 7 days. Then, the seedlings were watered with complete nutrient solution until ready to be used, following a method modified from Soh et al. [17].

The 14-day-old seedlings with first two-regular-leaves formed, were then transferred to pots (23 cm (width) ×27 cm (length) ×33 cm (height)) filled with growing medium, allocating one Chinese kale seedling per pot.

2.2.4 Determination of Chinese kale growth

Growth parameters recorded were plant height and fresh weight, with

recordings being made when the plants were 7, 14, 21, 28, and 35 days post-transplant.

2.2.5 Experimental design

Five treatments were proposed using Complete Randomized Design (CRD). Each treatment included four replicates and each replicate contained five Chinese kale seedlings. The information for each treatment is as follows, T1: complete nutrient as positive control, T2: solution of bio-extract and filtered water at a ratio of 1:500, T3: solution of bio-extract and filtered water at a ratio of 1:250, T4: solution of bio-extract and filtered water at a ratio of 1:100, T5: plain filtered water, as negative control.

2.2.6 Statistical analysis

All experiments were carried out in triplicate or higher. Statistical analysis was performed using Statistical Package for Social Science (SPSS 10.0, for Windows Inc., Chicago, IL).

3. Results and Discussion

3.1 Physical appearance

General characteristics such as fishy odor and dark-brown color were expected in the bio-extract containing fish waste and pineapple peel at beginning of the experiment. However, the odor of the fermented mixture in this study became quite pleasant at the end of the first month; the mixture released an alcoholic smell which was similar to that of typical bio- extracts dominated by yeast, as yeast film could be seen on top of the mixture's surface from the beginning to the 150 day mark, and its color became darker over time. Degradation of the bio- extract was rather rapid. The size of starting materials had visibly shrunk and the oily material that was initially observed on top of the mixture become thinner. At the end of the second month, almost all of the starting materials, including floating oil, were dissipated into patches of oil.

However, the physical characteristics of the bio-extract were not significantly changed from 90 days onward. The characteristics of the bio-extract in this study, as mentioned above, were considered appropriate for plant growth and nontoxic to the area where it was applied, as evident by soil and water division i.e., a weak alcoholic acidic smell and absence of gas bubbles.

3.2 pH

As indicated in Fig. 1, the pH of the bio-extract was determined during fermentation. The bio-extract ingredients were a combination of POME, fish waste, and pineapple peel, which were considered to be sources of acid; the pH of the mixture was indicated as mildly acidic. The pH declined during the first month of fermentation, indicating that metabolites produced by *in situ* micro- organisms were mainly acidic substrates.

After the first month of fermentation, the bio-extract pH was almost unchanged ($p > 0.01$) and remained at approximately 4.0, an indication to the activity of proteolytic enzymes from the fish waste, and produced by *in situ* micro-organisms, resulting in ammonium salts. The ammonium salts self-neutralized organic acids in the bio- extract preventing further acidification. However, a pH around 4.0 is considered to be strongly acidic, which promotes the absorption of some metallic elements in plants, especially aluminum, copper, iron, and boron, but inhibits plants absorbing certain nutrients such as phosphate and molybdenum [19]. For the reasons mentioned above, the bio- extract product in this study might be applicable in agricultural farms like organic farming, especially where soil quality is rather alkaline.

3.3 Electrical conductivity (EC)

Mineral content and total dissolved solid (TDS) were generally recorded in terms of electrical conductivity (EC).

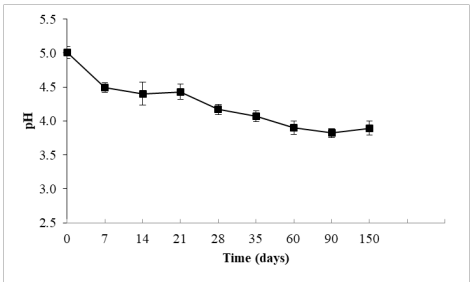

Fig. 1. Changes in pH of fish-pineapple bio extract over five months of fermentation.

The results showed that during the first four weeks of fermentation, EC increased rapidly from 1.5 to 5.7 ds/m (Fig. 2). This rapid increase in EC indicated that organic materials in the bio- extract were broken down into inorganic substances, by in situ micro-organisms. Digestion of these organic substances was determined by a decrease in COD of the fermentation mixture, as illustrated in Fig. 3. COD was closely related to EC, illustrated in Fig. 2. From the fourth week until the end of the experiment, both the EC and COD of the bio- extract had slightly declined. The EC of the fish and pineapple waste bio-extract ended up at 5.3 ds/m. It has been suggested that fish waste is mainly composed of proteins and carbohydrates; vitamins and trace minerals are considered to be minor components [20]. During bio-extract fermentation, the protein and carbohydrate content in fish waste underwent enzymatic degradation which released smaller molecules which further turned into small ions of organic acids. These organic acids consequently increased EC in the bio-extract [10].

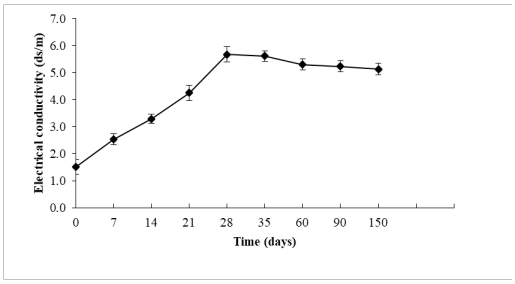

Fig. 2. Illustration of bio-extract electrical conductivity (EC) during 5 months of fermentation.

3.4 Reducing sugar and chemical oxygen demand

Since the sugars in pineapple are considered carbon sources available in bio- extract production, reducing sugar content could be a primary indicator of degradation of organic compounds in bio- extracts during fermentation. Together with COD, this indicates the actual amount of oxidizable hydrocarbon compounds in a bio-extract [10]. The change in both COD and reducing sugar content during fermentation illustrated in Fig. 3, showed that reducing sugar content (bar chart) declined from 66.3 to 2.3 g L^{-1} over the 150 days of fermentation. COD (line graph) also decreased, from 5300 to 200 mg L^{-1}. COD declined more sharply than reducing sugars did, especially during the first two weeks of fermentation. It was clearly seen that COD decreased by approximately 95% of its original value during the 150 days of fermentation. A suggestion was proposed on the difference in reduction of COD and reducing sugars; during the first two weeks, ethanol generated in the bio-extract probably turned into aldehyde compounds which might be included when measuring the level of reducing sugars. Alcohol turning into aldehydes is common in the fermentation of sugar into alcohol [21].

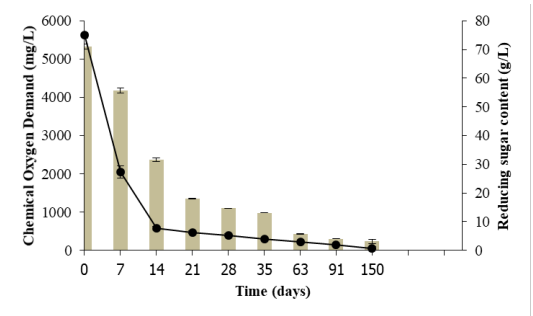

Fig. 3. Change in chemical oxygen demand (mg L^{-1}) (line graph) and reducing sugar content (g L^{-1}) (bar chart) in bio- extract during 150 days of fermentation.

3.5 Total carbon, total nitrogen, and C/N ratio

Table 2 shows the changes of total carbon (TC), total nitrogen (TN) and the C/N ratio in the bio-extract during fermentation, indicating that during fermentation, both carbon and nitrogen played significant roles in the bio-extract fermentation. TC decreased slowly during fermentation, by the end (150 days), TC had only slightly altered ($p > 0.01$). The high C/N ratio indicated that at the beginning of fermentation, the bio-extract was mainly composed of carbohydrates such as sugars and dietary fibers. A decline of carbon indicated that micro-organisms in the bio-extract preferred to digest carbohydrates rather than proteins. Whenever carbohydrates were metabolized by the micro-organisms, the carbon would leave the bio-extract in the form of CO2. On the other hand, as proteins in bio-extract were metabolized, ammonia and carbon dioxide would be emitted. However, carbohydrate reduction was a bit quicker than protein reduction, therefore the C/N of the bio-extract fell over the course of incubation [15]. Finding heterofermentative lactic acid bacteria in the bio-extract was one of the pieces of evidence that carbohydrate reduction was occurring in the bio-extract. These bacteria are able to produce lactic acid and ethanol using glucose as a carbon source, releasing carbon dioxide as a byproduct [22].

As shown in table 2, the total nitrogen detected in the bio-extract increased continually during the 5 months of fermentation. On day 60 of fermentation, the total nitrogen content in the bio-extracts continued to increase gradually until the end of fermentation ($p > 0.01$) as indicated in table 2. In this study, the total nitrogen content from the start to day 150 of fermentation remained within the acceptable range set by Thai standards for liquid biofertilizer [23]; these standards state that the total nitrogen content is required to be less than 3% by weight. The C/N ratio is typically used as a parameter indicating bio-extract maturity and stability. Moreover, the C/N ratio may be used to determine the progress of organic decomposition as well. For this reason, the C/N ratio was used as one of the parameters measured to determine the stage of bio-extract degradation. As is indicated in table 2, the C/N ratio fell by more than 50% in the first two weeks, from 61.30±0.62 to 29.80±0.50 and then continued to fall to 20.90 ± 0.32 by the end of the fifth month of incubation, approximately 30% of the initial value, indicating the stabilization of organic matter degradation.

3.6 Organic acid and ethanol production

Thai farmers usually consider bio-extracts as a type of liquid bio-fertilizer, and apply it similarly, as a way to promote plant growth. This categorization is partially true but bio-extracts provide less mineral nutrients than bio-fertilizers, manure for example. During fermentation, several byproducts were generated, such as acetic acid and butyric acid, and small-molecular-weight alcohols such as ethanol (Fig. 4). These small molecule products are considered essential nutrients for plant growth by Thai farmers. However, these metabolites have been proven to hinder the beneficial effects of the bio-extract itself, [24].

Table 2. Changes of total carbon, total nitrogen and C/N ratio in fish-pineapple bio-extract at different time of fermentation during 150 days.

Time(days)	Total carbon (%W/V)	Total nitrogen (%W/V)	C/N Ratio
Day 0	58.24±0.65[a]	0.95±0.25[a]	61.30±0.62a
Day 14	56.23±0.48[a]	1.85±0.11[b]	30.40±0.06[b]
Day 35	55.98±0.62[a]	1.88±0.22[b]	29.80±0.50[c]
Day 60	53.58±1.85[b]	2.24±0.31[c]	23.90±0.42[d]
Day 90	50.26±1.95[b]	2.28±0.22[c]	22.00±0.25[d]
Day 150	48.24±0.84[c]	2.31±0.45[d]	20.90±0.32[e]

Note: means followed by same superscripts indicate no-significant difference within the same column ($p > 0.05$) Values are means ± SD. Different letters in the same column indicate significant treatments difference ($p < 0.05$).

Therefore, the metabolites of focus in this study included acetic acid, propionic acid, butyric acid, lactic acid, and ethanol. Ethanol presented as the second most abundant metabolite in this study, after acetic acid (Fig. 4) which indicated a change in selected metabolites during fermentation. Shown in figure 4, during the five months of incubation, acetic acid, butyric acid, propionic acid, lactic acid and ethanol were found at rather high concentrations as alcohol would tend to be reduced to acetic acid by bacteria. Yeasts and fungi were dominant, indicated by the formation of a thin white film during the first month of fermentation. The formation of ethanol was an indication of the presence of yeasts and fungi in the bio-extract; the ethanol concentration rapidly increased during the first 35 days of incubation, and at the end of the month it had reached 189 mM.

However, from the second month onwards, the concentration of ethanol remained constant at about 257 mM. Lactic acid was the third major product in the bio-extract, after acetic acid and ethanol. The lactic acid concentration rapidly increased during the first two weeks of incubation and then stayed constant onwards (Fig. 4), suggesting that certain bacteria might have been metabolizing the lactic acid from the third week and on. Additionally, acetic acid reached a peak concentration of 165 mM at around 60 days of fermentation (Fig. 4). Among these organic acids, acetic acid, butyric acid, and propionic acid have been shown to promote root elongation, however butyric acid is considered rather toxic to plant leaves. Additionally, propionic acid can protect against certain pathogens, like viruses and bacteria, from infecting the plant [25].

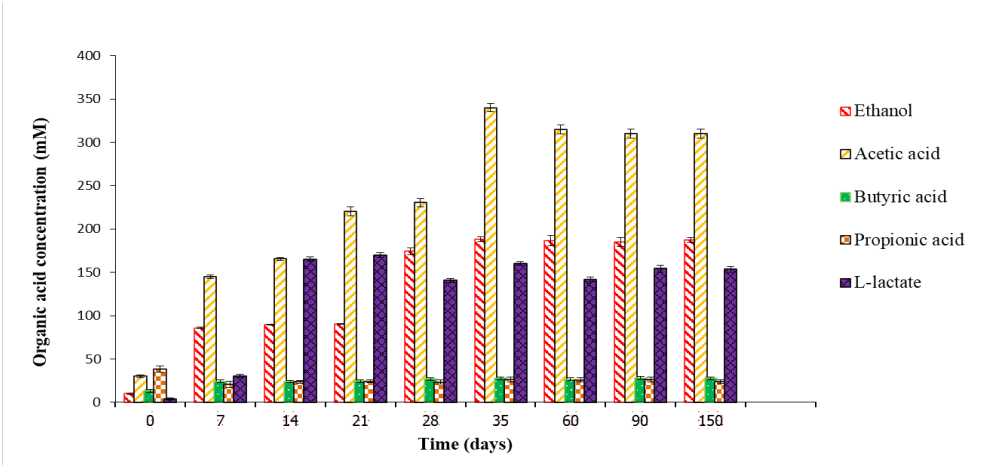

Fig. 4. Some organic acids and alcohol detected during bio-extract production for 5 months: ethanol (), acetic acid (), butyric acid (), propionic acid (), and L-lactate (). (bars represented as mean ± standard deviation from 3 determinations).

Table 3. Total Bacterial and Lactic acid Bacterial Cell Count during bio-extract fermentation.

Time of incubation (days)	Total bacteria (CFU)	Lactic acid bacteria (CFU)
0	7.30×10^6	4.28×10^6
7	10.14×10^6	6.25×10^6
14	8.25×10^6	7.84×10^6
21	8.12×10^6	7.57×10^6
28	5.65×10^6	5.25×10^6
35	5.18×10^6	4.15×10^6
60	3.51×10^6	2.89×10^6
90	3.41×10^6	2.55×10^6
150	3.20×10^6	2.50×10^6

3.7 Biological properties

Bacterial cell count is a key parameter for evaluating a bio-extract. The results in table 3 illustrate that throughout the 150 days of incubation, total bacteria increased rapidly in the first week, from 7.30×10^6 CFU to a maximum of 10.14×10^6 CFU by the end of the first week, then decreased throughout the remaining duration of the experiment, ending at 3.20×10^6 CFU, less than half of the initial amount.

The growth of lactic acid bacteria in the bio-extract with MRS medium was also measured during incubation. The cell number started at 4.28×10^6 CFU, peaked at 7.84×10^6 CFU on the fourteenth day of incubation, then rapidly fell afterwards, similar to the other bio-extract. At the end of experiment, lactic acid bacteria were measured to be at about 2.50×10^6 CFU.

It was clearly observed that during fermentation, the number of both total bacteria and lactic acid bacteria increased and then declined in the same way. Proliferation of microbes was in sync with the decline of reducing sugar, as indicated in Fig. 3, from the second week onward. After that, the levels of total bacteria and lactic acid bacteria declined, similar to the level of reducing sugar. Secondary metabolites are thought to have been generated from the metabolization of reducing sugars, and these metabolites probably inhibited the growth of bacteria in the bio-extract. The presence of hexoses such as glucose, and several secondary metabolites, i.e., acetic acid, CO_2, diacetyl, and acetoin, have been reported during metabolism of lactic acid by bacteria [25].

3.8 Qualities of bio-extract

Since the principal starting materials used for the production of bio-extract are of considerable interest for characterizing the qualities of the resulting bio-extract, table 4 indicates the qualities of the bio-extract at one-month post-fermentation. It could be concluded that the composition of the bio-extract, including nitrogen (N), phosphorus (P), and potassium (K), as well as organic acids, i.e., acetic acid and butyric acid, were at levels acceptable by the Thai standards for liquid biofertilizers, as illustrated in Table 4. This bio-extract could be employed as biofertilizer when diluted to appropriate concentrations.

Table 4. Characteristics of bio-extract at 1 month post fermentation as compared to the Standard liquid bio-fertilizer designated by Ministry of Agriculture and Cooperatives (2015) [23].

Parameters	Bio-extract	Standard liquid bio-fertilizer
pH	4.10±0.10	≤5
Electrical conductivity (ds/m)	5.70±0.22	≤10
Nitrogen (N, %, w/v)	1.25±0.14	≤3, bio-extract of animal origin
Phosphorus (P, %, w/v)	0.88±0.02	≤2, bio-extract of plant origin
Potassium (K, %, w/v)	1.56±0.10	≥1
Arsenic (As, %, w/v)	0	≥1
Cadmium (Cd, %, w/v)	0	≤50 mg/kg

Chromium (Cr, %, w/v)	0	≤5 mg/kg
Lead (Pb, %, w/v)	0	≤300 mg/kg
Mercury (Hg, %, w/v)	0	≤500 mg/kg
Copper (Cu, %, w/v)	0.01±0.00	≤2
Ethanol (mM)	125±2	≤500 mg/kg
Acetic acid (mM)	253±15	-
Butyric acid (mM)	24±1	-
Propionic acid (mM)	28±0	-
Lactic acid (mM)	125±1	-
Total bacteria (cfu/ml)	$1.7x10^5$	-
Lactic acid bacteria (cfu/ml)	$1.2v10^5$	-

3.9 Effect of bio-extract on Chinese kale growth

The bio-extract quality was further tested on Chinese kale growth. It was diluted with filtered tap water at 5 different ratios in order to find the optimal dilution factor. Chinese kale seedlings at the age 7-days after germination were used. Plant height and fresh weight were measured weekly for 5 weeks. Data illustrated in Table 2 indicates that there was no significant ($p > 0.01$) difference in the height of Chinese kale for the first seven days of cultivation, indicating that the plants were too young to have a response to treatment. At 35 days of cultivation, there was no significant difference between plants growing in complete nutrient solution and in the bio-extract at 1:100 dilution ($p > 0.01$). However, these two plant groups grew significantly better ($p < 0.01$) than those in the other groups, which were grown in bio-extract at dilution ratios of 1:500 and 1:250, and in plain filtered water. Plants grown in complete nutrient solution grew the tallest (35.62 cm), followed by bio-extract dilution of 1:100, 1:500, and then 1:250, with average heights of 35.55, 32.64, and 32.15, respectively.

The total fresh weight of Chinese kale was measured at 7 and 14 days of cultivation. Plants grown in complete nutrient solution had a higher fresh weight than those of other treatment groups, with a highly significant difference ($p < 0.01$). At 21 and 28 days of cultivation, plants grown in complete nutrient solution and in bio- extract at a dilution of 1:100 had a higher fresh weight than the other three, with a highly significant difference ($p < 0.01$). At the end of the experiment (35 days0, the plants grown in complete nutrient solution as well as those grown in bio-extract at the dilutions of 1:500, 1:250, and 1:100 had a significantly higher fresh weight ($p < 0.01$) than the plants grown in plain filtered water. The final average fresh weight, recorded at 35 days of cultivation were 90.47, 89.15, and 79.85 g for the 3 best treatments respectively, as shown in Table 6.

Table 5. Average height (cm) of Chinese kale grown in different dilution ratios of bio-extract and filtered water at different periods of growth for 35 days.

Treatments	Cultivation (d)				
	7	14	21	28	35
Complete nutrient solution	6.25^a	15.83^a	25.17^a	28.26^a	35.62^a
BE:: FW (1:500)	6.19^a	11.23^c	21.54^b	24.18^b	32.64^b
BE:: FW (1:250)	6.02^a	14.65^b	22.87^b	23.54^b	32.15^b
BE:: FW (1:100)	6.14^a	15.25^a	24.48^a	27.95^a	35.55^a
Filtered water	6.15^a	9.36^d	9.57^c	9.48^c	9.35^c
F-test	ns	**	**	**	**
CV%	11.23	18.32	16.45	15.48	16.58

BE: Bio-extract, FW: filtered water
ns = no significant statistical difference,
** = significant statistical difference at 99%.
Means within the same column followed by the same superscript letter were not significantly different at $p < 0.01$ by DMRT

Table 6. Plant stem and leaf fresh weight (in grams) of Chinese kale grown in different dilution ratios of bio-extract and filtered water during cultivation for 35 days.

Treatments	Cultivation (d)				
(Bio-extract and dilution ratio with water)	7	14	21	28	35
Complete nutrient solution	1.95[a]	10.55[a]	31.44[a]	75.34[a]	90.47[a]
BE: FW (1:500)	1.03[b]	5.25[b]	25.12[c]	69.12[b]	78.25[b]
BE: FW (1:250)	1.05[b]	5.25[b]	28.36[b]	69.34[b]	79.85[b]
BE: FW (1:100)	1.05[b]	5.58[b]	30.51[a]	74.25[a]	89.15[a]
Filtered water	0.91[b]	4.95[b]	4.95[d]	5.12[c]	7.56[c]
F-test	**	**	**	**	**
CV%	18.19	21.25	35.12	35.13	25.64

** = significant statistical difference at 99%.

Means within the same column followed by the same superscript letter were not significantly different at $p < 0.01$ by DNMRT. Values illustrated as means. Different letters in the same column indicate significant treatments difference ($p < 0.05$).

From Table 5 and 6, bio-extract diluted with filtered water at a ratio of 1:100 was considered the best treatment for Chinese kale and resulted in a similar growth rate to that of the plants grown with the complete nutrient solution. The bio-extract did not only supply major nutrients like nitrogen, phosphorus and potassium, it also provided several minor essential nutrients including vitamins and plant hormones. Several studies have mentioned that nitrogen insufficiency could retard plant growth or even lead to irreversible dwarfing of the plant, whereas deficiency of phosphorus and potassium are not reported to have as much of an effect on plant size as nitrogen deficiency does, but deficiency of these two elements does cause the plant severe weakness [26]. In addition, the pH of filtered water used to dilute bio-extract also plays an important role in plant growth; plants normally grow best at a pH of around 6.8. However, several studies have indicated that the application of bio-extract alone, without any addition of chemical fertilizers, did not promote growth in plants like cotton (*Gossypium* spp.). In a study on soil free culture of French marigold plants (*Tagetes erecta*), the application of bio- extract resulted in increased stem length and diameter during the first three weeks, as compared to those grown in complete nutrient solution; however, the plant stem length and diameter did not differ afterwards [14]. The reason for this was that the macronutrient and micronutrient contents of the bio- extract were not sufficient for the growth of these plants, especially in the later weeks of culturing [18]. Khaliq et al. (2006) [27] also showed that using sole EM (effective microorganism), a product similar to bio-extracts, on cotton plants did not increase the yield of cotton. Contrary to this, it was shown that EM promoted the growth of oyster mushrooms (*Pleurotus ostreatus*), increasing the growth by approximately two to four times more than that which was observed in plants where EM was not applied, suggesting that EM may benefit species of fungi more so than plants [28].

Fish waste was chosen as a bio-extract ingredient because it harbors groups of beneficial microorganisms, namely plant growth promoting bacteria (PGPB) which work in symbiosis with plants, doing things like producing growth regulators: cytokinins, gibberellins, ethylene, auxin, and indole acetic acid which all promote host-plant cell division and differentiation; PGPB also release several chemicals to reduce the harmful effects of phyto-pathogens. PGPBs living outside host plants, on the other hand, provide other benefits such as processing various nutrients inaccessible to the plant into a form that is accessible to it, like turning insoluble phosphorus into a soluble form-orthophosphate, or turning gaseous nitrogen into nitrate [29]. In one study, it was shown that PGPB were able to produce plant growth regulators like indoleacetic acid (IAA), gibberellic acid, cytokinin, and ethylene, a gaseous plant hormone [30].

In this study, the bio-extract dilution ratio most suitable for plant growth was 1:100. The low pH of the bio-extract did not affect the growth of Chinese kale, since Chinese kale growth is hindered in mildly acidic conditions (pH 5.5-6.8) [31]. Moreover, when the bio-extract was diluted to 1%, acidity was weakened and the Chinese kale was no-longer impaired by bio-extract application.

This study revealed that bio-extract at a dilution ratio of 1:100 provided an inadequate amount of essential nutrients to Chinese kale seedlings at 7-14 days of cultivation, as the resulting fresh weight was lower than the complete nutrient solution treatment group. However, at the later stage, the plants grown in bio-extract resumed growth and eventually outgrew the plants grown in complete nutrient solution, as the plants grown in both media had fresh weights which were not significantly different ($p > 0.01$) at 21 and 28 days of cultivation. However, there was no significant difference in plant height between the plants grown in complete nutrient solution and those grown in bio-extract at dilution 1:100 (Table 5). From the reasons above, utilization of bio-extract for cultivation can be considered a lower cost and more environmentally friendly tool than chemical fertilizers. Utilization of bio-extract is also an appropriate way to re-use agricultural waste. These results indicate that the addition of POME into bio-extract fermentation could be practical, especially when applied together with fish waste and pineapple peel.

4. Conclusions

This mixture of POME, fish waste, and pineapple waste could be efficiently applied as ingredients for bio-extract production. In order to produce a high quality bio-extract, the mixture is required to be fermented for one to two months. According to the Thai standard for liquid bio-fertilizers, this bio-extract is best after undergoing one month of fermentation. The bio-extract product of two-months of fermentation or longer was also applicable, however it is considered too concentrated to be used as a bio-fertilizer. For this reason, the bio-extract requires an appropriate dilution before applying it as a biofertilizer for horticulture. The bio-extract in this study provided a high level of nutrients, EC, and low pH. The pH of the bio-extract in this study is considered suitable for growing Chinese kale; however, the nutrient content in the bio-extract was still too low for this plant. Among the dilution ratios (1:500, 1:250, and 1:100), 1:100 was the most effective for growing Chinese kale. For further studies, POME should be applied to different crops, such as Morning glory, for wider usage.

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