The study on multi-stage bio-extract production from wastewater originated from virgin coconut oil manufacturing process with fish wastes

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ABSTRACT

Recently, Bio-extract has gained popularity as alternative to chemical fertilizers and pesticides in agriculture practices. The qualities of bio-extract, however, are different depending on the types of raw materials and methods of production. The utilization of low quality bio-extract may affect plant growth and soil qualities. The objective of this study was to investigate the effect of supplements on the qualities of bio-extract produced from fish waste in the multi-stage production process. For the first stage, fish waste and wastewater from virgin coconut oil manufacturing process at 3:1 ratio (by weight) 20% molasses were mixed and incubated at room temperature for 30 days. For stages 2 and 3 fermentation, sludge obtained from the first stage was subsequently supplemented with molasses and bio-extract obtained previously (either 1st or 2nd stage) at different levels. Therefore, 4 treatments were resulted: no supplements (1), 20% molasses (2), 10% bio-extract (3) and 10% molasses and 10% bio-extract (4). Results showed that the physical changes (i.e., color and texture), trace elements, microbial evolution and COD reduction of treatment 1 for stages 2 and 3 were significantly lower than those of treatments 2, 3 and 4 ($P<0.01$). The physical changes, pH, and microbial evolution of treatment 2 were insignificantly different from those of treatments 3 and 4; however, the percentages of organic matter degradation expressed as COD reduction of the treatment 2–53.17% and 30.50, for stages 2 and 3, respectively–were significantly higher than those of treatments 3 and 4 ($P<0.01$). Results further revealed that the qualities of bio-extract were of the order of 3rd < 2nd < 1st stage. Results suggested that qualities of bio-extract production using fish waste as raw materials declined as cycle of fermentation increased. Thus, if repeated fermentation is intended suitable supplements should be introduced to ensure the final bio-extract qualities.

Keywords: Bio-extract ,Wastewater Originated from Virgin Coconut Oil Manufacturing Process, Fish wastes , Molasses and Multi-stage

1. INTRODUCTION

Bio-extract is a fermented product obtained from the degradation process of plant or animal waste supplemented with appropriate carbon sources by aerobic and/or anaerobic microorganisms [1]. Bio-extract consists of microorganisms, nutrients, organic acids, amino acids and plant hormones [2, 3]. In Thailand, production of bio-extract has been passed down from generation to generation among local Thai people. Local farmers have been using bio-extract to reduce or replace chemical fertilizer, pesticide and insecticide usage for a long time.

Most bio-extract production in Thailand is produced at the household and community level. The fermentation process starts from mixing raw materials such as plant or animal wastes with molasses and water at different ratios, fermenting at room temperature for various time intervals such as 7-15 days, 75 days and then filtering before usage. Sludge and bio-extract obtained from the process of bio-extract production is used, respectively, as raw material and seeding/inoculum for production in the following stages. In subsequent fermentation, supplements, i.e., sugar, molasses and water, at different ratios will be added. Since the processes of bio-extract production are diversified and their effects on the quality of the bio-extract obtained from the process are poorly understood, the application of such bio-extract in agriculture may pose an adverse effect on either plants or soils. Therefore, this study aimed to investigate the effect of supplements on the qualities of bio-extract produced from fish waste using the multi-stage fermentation process. Physical, chemical and microbiological changes during the fermentation process and phytotoxicity based on the germination index were measured. The results gained from this study will benefit the development of bio-extract quality in order to meet the standard of Ministry of Agriculture and Cooperatives (MOAC) (2001) [5] and thus, be used without any adverse effects on plant growth.

2. MATERIALS AND METHODS

2.1 Materials

Raw materials used in this study and their chemical and biological characteristics are provided in Table 1. Wastewater originating from the virgin coconut oil (VCO) manufacturing process was prepared according to the method described by Tripetchkul *et al*. (2012) [4].

Table 1 Physico-chemical properties of raw materials used in this study.

Properties	Raw materials		
	Fish waste	VCO	Molasses
pH	5.84±0.02	4.09±0.02	4.64±0.01
Total solid (%)	36.23±2.62	6.57±0.28	46.81±0.78
Oil and grease (%)	nd	4.91±0.60	nd
COD (mg L^{-1})	nd	2021±139	nd
Total carbon (%)	43.79±1.90	5.93±0.61	53.74±1.12
Total sugar (%)	nd	nd	71.31±0.25
C/N ratio	4.49±0.58	25.45±1.54	119.82±5.78
Total nitrogen (%)	9.82±0.80	0.23±0.01	0.45±0.03
Potassium (%)	0.15±0.01	0.02±0.00	1.28±0.17
Phosphorus (%)	0.002±0.000	0.060±0.000	0.130±0.010
Total bacteria (CFU/ml)	nd	1.04×10^5	nd
Lactic acid bacteria (CFU/ml)	nd	5.9×10^3	nd

Note: nd= not determined.

2.2 Experimental procedures

The experiment was divided into 4 treatments (T_1, T_2, T_3 and T_4) and the control (T_C). Details of the experiment are shown in Table 2. In each treatment, samples were taken for physical, chemical and microbiological testing at day 30, 60 and 90.

Table 2 Experimental procedures

Treatments	Stage 1 (fermentation time 30 days)	Stage 2 (fermentation time 30 days)	Stage 3 (fermentation time 30 days)
T_C (Control)	FW + W_{VCO} + M (3:1:1) by weight, brewed at room temperature for 90 days		
T_1 (FW:W_{VCO})	FW + W_{VCO} + M (3:1:1) by weight, brewed at room temperature for 30 days	FW sludge from stage 1 + W_{VCO}(3:2) by weight	FW sludge from stage 1 + W_{VCO}(3:2) by weight
T_2 (FW:W_{VCO}:M)		FW sludge from stage 1 + W_{VCO} + M (3:1:1) by weight	FW sludge from stage 1 + W_{VCO} + M (3:1:1) by weight
T_3 (FW:W_{VCO}:BioE)		FW sludge from stage 1 + W_{VCO} + BioE (3:1.5:0.5) by weight	FW sludge from stage 1 + W_{VCO} + BioE (3:1.5:0.5) by weight
T_4 (FW:W_{VCO}:M:BioE)		FW sludge from stage 1 + W_{VCO} + M + BioE (3:1:0.5:0.5) by weight	FW sludge from stage 1 + W_{VCO} + M + BioE (3:1:0.5:0.5) by weight

Note: FW: Fish wastes, W_{VCO}: Wastewater Originated from Virgin Coconut Oil (VCO) Manufacturing Process, M: Molasses and BioE: Bio-extract.

2.3 Analysis

Physical characteristics of bioextract, i.e., odour, color and texture, were visually observed. The evolution of pH by pH meter (model Consort C830) and the chemical oxygen demand (COD) using the close reflux and titrimetric method [6] were determined. Total nitrogen (TN) was also quantified according to Macro Kjeldahl method [7]. Phosphorus was determined using Ascorbic Acid Method and potassium determined by Atomic Absorption Spectrophotometer [7]. Concentrations of ethanol and organic acids such as acetic acid, propionic acid, butyric acid and lactic acid produced during fermentation were closely followed using FID gas chromatography [8]. The total bacteria and lactic acid bacteria (LAB) were enumerated by plate count using, respectively, nutrient agar and MRS agar [9]. Phytotoxicity was tested against the Chinese cabbage (*Brassica pekinensis*) seed following the seed germination test. Then, the germination index was calculated using the method proposed by ISTA (1993) [10].

2.4 Statistical analysis

The experimental data was statistically analyzed. Analysis of variance and multiple comparison using Fisher's least significant difference (LSD) test, at the confidence interval of 99% were performed by SPSS software program version 15 (SPSS Inc., Chicago, USA).

3. RESULTS AND DISCUSSIONS

3.1 Physico-chemical and biological changes

<u>Physical appearance</u>

The physical appearance in terms of color, odor and general characteristics (oil & grease, film yeast and bubble gas on the surface of the bio-extract) at day 30, 60 and 90 of T_c complied with the standard of MOAC (2001) [5]. In stage 2 and 3, changes in physical appearance of T_1 without molasses addition suggested that degradation of fish waste was rather slow since no change in physical appearance were observed. In comparison with T_3 and T_4, the color of T_2 with molasses, FW sludge and VCO wastewater supplementation was more intense, blacker in tone while the odor was alcoholic. The results also revealed that if the fermentation time exceeded 3 months (stage 3), the physical appearance was not in good agreement with the desirable characteristics of bio-extract reported by the MOAC (2001) [5].

<u>pH</u>

The pH evolution during the multi-stage fermentation of bio-extract is shown in Figure 1. In T_1, T_2, T_3 and T_4, pH at the end of stages 2 and 3 were significantly higher than those of the control (T_C) (P<0.01). The results suggested that an increase in pH observed in all treatments may stem from the inadequate carbon source present in the system for biodegradation, resulting in a decrease in organic acid production. Consequently, acceleration of nitrogen mineralization of the FW sludge occurred, leading to ammonia production [11]. In general, the pH of bio-extract should be low (pH<4.5) in order to inhibit growth of pathogenic microorganisms present in the bio-extract [12] during production process.

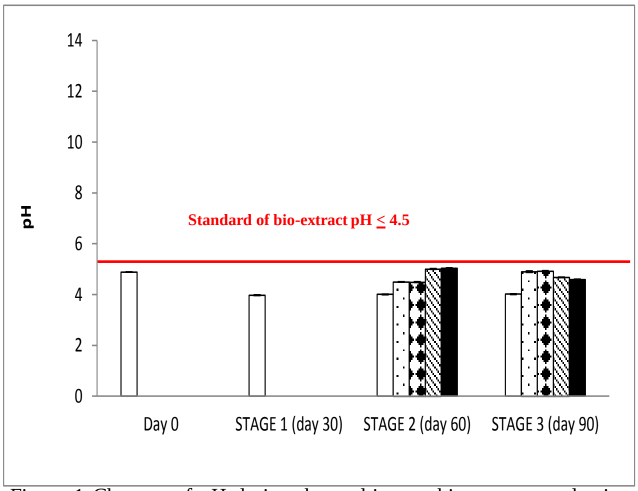

Figure 1 Changes of pH during the multi-stage bio-extract production (T_C; Control, T_1; SW:W$_{VCO}$, T_2; SW:W$_{VCO}$:M, T_3; SW:W$_{VCO}$:BioE, T_4; SW:W$_{VCO}$:M:BioE)

Total nitrogen (TN), phosphorus (P) and potassium (K)

Table 3 shows changes in total nitrogen, phosphorus and potassium during the whole period of multi-stage bio-extract fermentation. Results showed that for control (T_C), changes in TN, P and K from day 30 onwards were marginal and statistically indifferent (P>0.01). At day 60 and 90, the amounts of TN, P and K in T_C were significantly higher than those in T_1, T_2, T_3 and T_4 (P<0.01). The amounts of TN, P and K in the 4 treatments gradually reduced from stage 1, followed by stage 2 and stage 3, respectively. It is plausible that decline of TN, P and K maybe due to that fact that fish waste (FW) sludge, the raw material for multi-stage bio-extract production in stage 2 and 3, were partially decomposed (stage 1), resulting in the decrease in the amounts of TN, P and K of the FW sludge: the amount of the TN present in the FW sludge (1.36%) was much lower (about 7 times) than that found in the fish waste.

In stages 2 and 3 of bio-extract production, the TN and K contents found in T_2 (20% molasses addition) were significantly higher than those of T_1 and T_3 without molasses addition (P<0.01). The differences in P content of all treatments may stem from the high potassium and nitrogen content in the added molasses (Table 1). Results suggested that molasses plays an important role in biodegradation of fish waste in the multi-stage bio-extract production given that less than 25% of molasses addition (in T_1, T_3 and T_4) may retard the N-mineralization.

C/N ratio

In stage 2 and stage 3, C/N ratios of the 4 treatments containing the FW sludge from stage 1, VCO wastewater and either molasses, bio-extract or a combination of molasses and bio-extract were significantly higher than that of control (T_C) (P<0.01). Among all 4 treatments, T_2 was of the lowest C/N ratio comparing to treatments with a low ratio of molasses addition.

Table 3 Changes in nitrogen, phosphorus and potassium during the multi-stage bio-extract production.

Times / Treatments	Nitrogen (%) Stage1	Stage2	Stage3
T_C		1.07±0.03	1.05±0.03
T_1		0.67±0.01	0.48±0.02
T_2	1.04±0.02	0.93±0.01	0.65±0.02
T_3		0.86±0.01	0.63±0.02
T_4		0.79±0.01	0.61±0.02
Times / Treatments	**Phosphorus (%) Stage1**	**Stage2**	**Stage3**
T_C		0.002±0.001	0.002±0.001
T_1		0.002±0.001	0.002±0.001
T_2	0.001±0.001	0.002±0.001	0.002±0.001
T_3		0.002±0.001	0.002±0.001
T_4		0.001±0.001	0.001±0.001
Times / Treatments	**Potassium (%) Stage1**	**Stage2**	**Stage3**
T_C		2.96±0.02	2.57±0.03
T_1		1.96±0.17	1.46±0.05
T_2	3.12±0.01	2.28±0.26	2.17±0.22
T_3		1.19±0.10	1.38±0.07
T_4		2.55±0.21	2.18±0.15

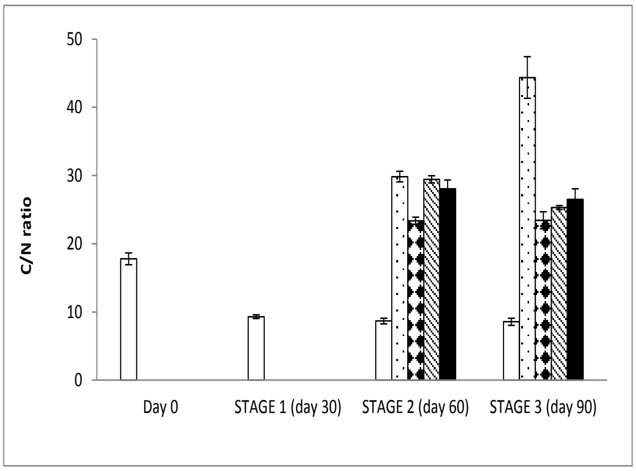

Figure 2 Trend of C/N ratio during the multi-stage bio-extract production (T_C; Control, T_1; SW:W$_{VCO}$, T_2; SW:W$_{VCO}$:M, T_3; SW:W$_{VCO}$:BioE, T_4; SW:W$_{VCO}$:M:BioE)

Ethanol and organic acids

Table 4 shows the changes in ethanol and organic acids concentration during the multi-stage fermentation of fish waste bio-extract. The results showed that, for T_2 and T_4, the concentration of ethanol, acetic acid and lactic acid in 2nd and 3rd fermentation stage was significantly higher than those of T_C, T_1 and T_3 without molasses addition (P<0.01) which may be responsible by the presence of readily available carbon contained in molasses [13] which microorganisms can utilize for their growth and decomposition of organic substances.

The evolution of total bacteria and lactic acid bacteria

Table 5 shows the evolution of total bacteria and lactic acid bacteria during the multi-stage fermentation of bio-extract. The results showed that in the latter 2 stages of bio-extract production, total bacteria and lactic acid bacteria of T_1, T_2, T_3 and T_4 decreased significantly (P<0.01) compared with that of control (T_C). This is possibly due to an appropriateness of environmental factors, especially the higher C/N ratio of mixing materials and pH. The optimum C/N ratio in the range of 25-30 is ideal for maximum decomposition [14]. The bio-extract with higher initial C/N ratio (Figure 2) would render decomposition by the microorganisms difficult. In addition, the bio-extract with higher initial pH (Figure 1) are favorable to growth of lactic acid bacteria [15].

Comparing with T_1 and T_2 without bio-extract supplementation, the number of lactic acid bacteria in T_3 and T_4 supplemented with bio-extract was significantly higher (P<0.01).

Table 4 Concentrations of organic acids detected during the multi-stage bio-extract production.

Times / Treatments	Ethanol (mM)		
	Stage1	Stage2	Stage3
T_C		211±12	200±13
T_1		0±0	0±0
T_2	242±30	416±12	410±21
T_3		0±0	0±0
T_4		141±12	127±26
Times / Treatments	**Acetic acid (mM)**		
	Stage1	Stage2	Stage3
T_C		459±3	422±2
T_1		491±17	508±29
T_2	458±9	854±16	862±30
T_3		201±9	219±9
T_4		906±31	832±33
Times / Treatments	**Lactic acid (mM)**		
	Stage1	Stage2	Stage3
T_C		182±2	159±2
T_1		205±4	196±5
T_2	187±2	248±3	248±2
T_3		220±2	212±2
T_4		239±2	237±2

Table 5 Evolution of both total bacteria and lactic acid bacteria during the multi-stage fermentation of bio-extract production

Times / Treatments	Total bacteria (Log cfu/ml)		
	Stage1	Stage2	Stage3
T_C		5.70±0.01	5.40±0.03
T_1		4.75±0.05	4.52±0.06
T_2	5.96±0.02	4.84±0.02	4.58±0.02
T_3		5.86±0.03	4.71±0.02
T_4		6.02±0.03	4.67±0.02
Times / Treatments	**Lactic acid bacteria (Log cfu/ml)**		
	Stage1	Stage2	Stage3
T_C		4.79±0.04	4.49±0.04
T_1		3.53±0.11	3.25±0.03
T_2	5.79±0.04	3.76±0.02	3.58±0.02
T_3		3.98±0.03	3.87±0.01
T_4		4.00±0.05	3.92±0.02

3.2 Performance of degradation

COD reduction

The chemical oxygen demand (COD) was selected as an indicator for the extent of degradation of organic and inorganic matter present in the mixture. For all 4 treatments, the reduction of COD in both bio-extract and FW sludge (Table 6) were significantly higher in stage 1, followed by stage 2 and stage 3. The COD reduction of FW sludge and bio-extract in T_2 was higher than those of T_1, T_3, T_4 and T_C (P<0.01). The results also revealed that the treatments with molasses addition (T_2 and T_4) was of significantly higher COD reduction than the treatments without molasses supplementation (T_1 and T_3).

3.3 Phytotoxicity

Germination index (GI) was used as an indicator of phytotoxicity in this study. GIs greater than 80% indicate a beneficial effect on seed growth, whilst values lower than 80% indicate potential phytotoxicity [16]. In Figure 7, T_1, T_2, T_3 and T_4 showed GI values higher than 80%, indicating a beneficial effect on bio-extract produced on the seed growth. T_2 and T_4 showed lower GIs in comparison with T_1, T_3 and T_C. It may be due to higher organic acid concentrations present in the bio-extract (Table 4). Some researchers reported that high concentration of ethanol and organic acids could inhibit elongation of roots [17, 18, 19]. Therefore, the bio-extract produced should be diluted appropriately prior to application as plant supplement to minimize adverse effects.

(Table 6) COD reduction of bio-extract and sludge during the multi-stage fermentation of bio-extract production

Times / Treatments	COD reduction of bio-extract (%)		
	Stage1	Stage2	Stage3
T_C		34.53±6.64	10.97±4.35
T_1		29.68±6.64	34.54±4.35
T_2	72.26±6.64	53.17±4.35	13.76±4.35
T_3		35.51±2.51	13.76±4.35
T_4		49.95±7.53	25.76±6.64
Times / Treatments	**COD reduction of sludge (%)**		
	Stage1	Stage2	Stage3
T_C		28.69±2.51	27.82±2.48
T_1		59.74±1.59	28.54±1.38
T_2	71.49±4.35	61.85±2.75	33.83±1.48
T_3		48.73±2.40	36.45±1.25
T_4		54.96±2.51	31.99±2.49

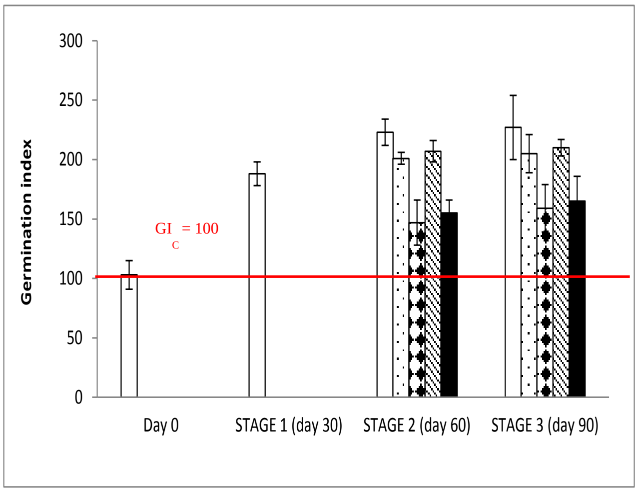

Figure 4 Germination index during the multi-stage bio-extract production (T_C; Control, T_1; SW:W_{VCO}, T_2; SW:W_{VCO}:M, T_3; SW:W_{VCO}:BioE, T_4; SW:W_{VCO}:M:BioE)

CONCLUSION

The FW sludge obtained from the fermentation of fish waste bio-extract together with VCO wastewater (stage 1) can be used as raw material for bio-extract production in further stages. For the multi-stage fermentation of fish waste bio-extract production, either molasses at concentration of 20% or molasses at concentration of 10% together with 10% bio-extract were required in order to meet the desirable characteristics of bio-extract specified by the standard of MOAC (2001). The FW bio-extracts produced could be used for agricultural activities without any adverse effect on plants. In order for the bio-extract produced to be of highest quality, multi-stage fermentation of the mixtures should not exceed 3 months, 1 month for each stage.

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