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STUDY ON BIOSYNTHESIS OF LACCASE ENZYME FROM FUNGI AND APPLICATION OF HYDROLYSIS OF TEA LEAVES TO OBTAIN BIOACTIVE POLYPHENOLS

Vu Dinh Giap[1,*], Bui Thi Tuyet Xuan[2], Khuat Thi Minh Hien[3]

ABSTRACT

Laccase (EC 1.10.3.2) is a poly copper oxidase found in plants, fungi, and bacteria. In this study, 21 fungal strains were screened for laccase activity, strain *Clitopilus* sp. BV8 showed the highest activity at 7,943.1 U/L. Fungal strains were fermented for 7 days on medium containing the following components: $MgSO_4$ (0.5 g/L); KH_2PO_4 (1.5 g/L); glucose (5 g/L); pH 6.0, tomato (2%), urea (2 g/L) and cultured at 25°C under the condition of continuous shaking at 200 rpm, the highest laccase activity was 65 U/mL. Crude laccase enzyme was concentrated from the culture solution with optimum temperature and pH at 40°C and pH 5.0, respectively. Total polyphenol content released and antioxidant activity from green tea leaf extract supported by laccase enzyme were also evaluated. The results showed that in optimal conditions for hydrolysis of tea leaves in 18 hours, the enzyme/substrate ratio was determined to be 5.0 U/gds at pH 5 at 40°C. Total polyphenol content and antioxidant activity reached the highest values of 136.15 mgGAE/g and 78.05%, respectively.

Keywords: *Tea leaves, polyphenol, antioxidant activity, laccase, oxidative enzymes.*

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1. INTRODUCTION

Laccases, which are versatile blue polyvalent oxidases with the capacity to oxidize a diverse range of both aromatic and non-aromatic compounds, are frequently studied in fungi due to their superior redox potential compared to that of bacteria and plants [1]. Numerous filamentous fungi isolate with elevated laccase biosynthesis have been identified, including, such as *Trametes versicolor* [2], *Streptomyces cyaneus* [3], *Melanocarpus albomyces* [4], *Trametes modesta* [5]. Many white rot fungi, in particular, are engaged in lignin metabolism. Laccase is produced by *Pycnoporus cinnabarinus* as a lignin-degrading enzyme, while laccase is produced by *Pycnoporus sanguineus* as a phenol oxidase. Laccase is a polyphenol oxidase whose active site is rich in copper. Laccase is able to oxidize a variety of structures that do not contain phenolic groups and polyphenolic compounds by utilizing small molecules as intermediate electron carriers [6]. Most people are interested in the polyphenol chemicals found in tea leaves. Polyphenols are substances that are found naturally in plants and are very powerful antioxidants. Tea leaves contain polyphenols that are significantly distinct from those in other plants. Catechin compounds make up the majority of the primary ingredients. Green tea catechins, which have potent antioxidant properties, have been employed extensively in functional meals up till now. Free radicals may be eliminated by natural antioxidants like polyphenol compounds, which is an extremely efficient way to stop oxidation [7]. The body is protected by polyphenols against several ailments brought on by free radicals. They include aromatic rings with one, two, three, or more hydroxyl groups (OH) immediately linked to the benzene ring in their

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molecules. The physicochemical attributes or biological activity vary depending on the amount and placement of the -OH groups within the chemical structure. Polyphenols in the cell wall of green tea leaves are often locked together by hydrogen bonds and der Waals bonds, making them difficult to dissolve in water and absorb [8]. To separate these polyphenols, synergistic oxidizing enzymes such as laccase and lignin peroxidase must be used to break these bonds and liberate the polyphenols into smaller, more water-soluble molecules. When oxidizing enzyme preparation is introduced to hydrolyze green tea leaves, it acts on the polyphenols by converting them to enzyme intermediates such as reactive radics (which alter by adding or withdrawing an electron). These intermediates then act on other polyphenols to disrupt their bonds. Other extraction and purification processes were used to get the polyphenols emitted by this network. The enzyme-assisted extraction approach refers to the use of enzyme laccase from the *C. prunulus* BV18 fungus to increase the effectiveness of polyphenol extraction in green tea leaves [9, 10]. The enzyme precisely breaks down the structural components enclosing the plant cells, allowing the solvent and extract to come into touch more easily. Methods of extracting bioactive substances from tea leaves require cell and membrane disruption, and enzymes such as laccase can help strengthen them [11]. Extraction of bioactive compounds from tea leaves. To add originality to the study of deriving biologically active substances from plants, we conducted a study on the application of laccase enzyme to the degradation of green tea leaves in order to obtain bioactive polyphenols.

2. MATERIALS AND METHODS

2.1. Materials

Ba Vi National Park (Ha Noi, Vietnam) was the source of the isolation and purification of 21 studied fungal isolates. They are kept in the laboratory, HaUI Institute of Technology, Hanoi University of Industry. Tea leaves (*Camellia sinensis*) were obtained from Thai Nguyen province (Vietnam). Samples were dried to constant weight at 60°C, ground into powder and stored in desiccant bags, and kept at a cool and dry place.

2.2. Enzyme assay

Laccase activity was determined by the ABTS oxidation method (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) The reaction solution consisted of 50 µl enzyme solution, 100 µl sodium acetate buffer (100 mM, pH). 5), 50 µl ABTS (10 mM) Samples were incubated at 40°C for 30 minutes, spectrophotometrically measured at 420 nm [12].

2.3. Determination of suitable culture conditions for laccase

Effect of carbon and nitrogen sources

The initial basal medium includes $MgSO_4$ (0.5 g/L); KH_2PO_4 (1.5 g/L); glucose (5 g/L); yeast extract (2 g/L); pH 6.5; and supplement (2%) of different substrate sources: Straw, corn, potato, tomato, and soybean. Meanwhile, yeast extract nitrogen for fungal growth from basic culture medium was substituted and other sources tested including (2 g/L): KNO_3; $(NH_4)_2SO_4$; peptones; $NaNO_3$; and urea. Fungal strains are fermented at 30°C, from 1 to 9 days, shaking continuously at 200 rpm. Laccase activity was determined from fungal culture.

Effect of temperature and pH

Fungal strains were cultured on a basic culture medium (pH 6.5) supplemented with substrate (2%) and a suitable nitrogen source. The optimum temperature and pH of the laccase were determined by measuring the enzyme activity according to the method described with the temperature varying from 20 to 40°C and the pH from 3.0 to 8.0.

2.4. Characterization of enzymes

The crude enzyme solution obtained after liquid fermentation was further precipitated with two types of solvents. The aliquot was precipitated with ethanol solvent and ammonium sulfate inorganic salt with concentrations ranging from 40 to 70%, and then centrifugation (10 min, 4000 rpm) collected protein residue. The fractions obtained after centrifugation, protein residues will be

washed and remixed with 100 mM sodium acetate buffer (pH 5.0), continue to centrifuge to collect protein residues, and repeat 2 - 3 times. The effect of pH on laccase activity was tested with pH from 4.0 to 8.0 in 100 mM sodium acetate buffer (pH 4.0 - 5.5) and 100 mM sodium phosphate (pH 6.0 - 8.0). The optimum temperature was determined at 30 to 60°C on ABTS substrate in sodium acetate buffer (100 mM) at pH 5.5.

2.5. Determining suitable conditions for extracting tea leaves by laccase

Tea leaves (5.0 gram per reaction) were milled for 5 minutes to powder, added water (1 : 5, w/v) and sonicated at 40^0C for 10 min. And then, the samples were incubated with laccase enzyme (10 U/gds) from *Clitopilus s*p. BV8 at 40°C and monitored at 2, 18, 21, 24, 42 and 48 hours. Moreover, the effects of different laccase activity [U/gds, U of enzyme laccase per gram dried tea leaves (substrate)] were investigated, including 1.0, 2.0, 3.0, 4.0, and 5.0 U/dgs (laccase) at 40°C, pH 5.0 during 18 hours. The reaction was carried out in 100 mM MOPS buffer (pH 5.0), and for comparison purposes, a control with heat - inactivated enzyme (95°C for 30 min) was used. The hydrolyzate solution was filtered with Whatman filter paper No.41 and centrifuged for 30 minutes at 10,000 rpm. The supernatant extract was collected and dried at 50°C. The obtained samples were analyzed for their total polyphenol content and antioxidant activity.

2.6. Determination of total polyphenol content

Polyphenols in the extract were determined by colorimetry using the Folin - Ciocalteu reagent. This reagent contains a phosphor - tungstenic acid oxidizing agent, during reduction, phenol hydroxy groups are easily oxidized, this oxidizing agent produces a blue color with maximum absorbance at 765 nm. The polyphenol content in the sample is proportional to the sample strength and is calculated as gallic acid. The reaction mixture consists of 1 ml of sample to be quantified, 6 ml of distilled water, and shaken well. Then add 0.5 ml Folin-Ciocalteu reagent 10%, shake well, and let stand. After 5 minutes, add 1.5 ml Na_2HCO_3 20%, shake well. Add distilled water to a volume of 10 ml. Keep test tubes in the dark for 2 hours then measure the absorbance of all samples on a prepared spectrophotometer at 765 nm [13].

2.7. Determination of antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of an extract sample was determined using the method described by Brand-Williams *et al.* (1995) [14]. The sample of desiccated extract was dissolved in DMSO (100%). DPPH was combined with 96% ethanol. The absorbance of a sample was measured at 515 nm using an ultraviolet spectrophotometer. The free radical scavenging capacity (SC, %) of the extract was calculated as follows [14]:

$$SC\ (\%) = \left[100 - \frac{Abs(test) - Abs\ (blank)}{Abs\ (negative)} \times 100\right] \pm \sigma$$

The standard deviation (σ) is calculated by Duncan's formula as follows:

$$\sigma = \sqrt{\frac{(\sum x_i - \bar{x})^2}{n-1}}$$

3. RESULTS AND DISCUSSION

3.1. Screening for fungi with high Laccase activity

The ability of 21 fungal isolates to biosynthesize Lac enzyme was measured by their ability to oxidize ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid, Sigma). The results of determining the laccase activity of 21 fungal strains after centrifugation to remove biomass and impurities are presented in table 1.

The screening findings for laccase activity indicated that, 8 of the total 21 strains exhibited activity, equivalent to 38% with activity from 40.6 to 7,943.1 U/L. In which, some strains showed high activity such as *Polystitus* sp. BV5 (454.7 U/L) and *Nemania* sp. BV17 (564.2 U/L). In particular, the strain *Clitopilus* sp. BV8 (7,943.1 U/L) exhibited the highest activity.

Related to this research direction, Thuy *et al.* (2015) isolated and characterized the morphological characteristics of 5 mold strains (BN1, BN2-1, BN2-2, DA3-1 and BV1) with active

laccase from 1,480 to 2,472 U/L. Which, strain BV1 belongs to the genus *Meruliporia* sp. and is the strain with the highest enzyme biosynthesis ability reaching 2,472 U/L and the highest ratio of enzyme activity to dry weight after 5 days of culture (E/M) of 54.04 U/mg [15]. According to Mai *et al.* (2012)[16], *Trametes versicolor* fungus used glucose substrate as an inducer, and the highest enzyme activity was obtained at 4,250 U/L after 7 days of culture at shaking speed 200 v/p, pH 6.0 [17]. Thus, in this study, the strain *Clitopilus* sp. BV8 showed high activity compared with some remaining strains to be studied further.

Table 1. Laccase enzyme activity of fungal strains

TT	Fungal strains	Laccase (U/L)
1	*Auricularia* sp. BV1	137.5 ± 0.2
2	*Ganoderma* sp. BV2	0
3	*Trametes* sp. BV3	0
4	*Bisporella* sp. BV4	0
5	*Polystitus* sp. BV5	454.7 ± 0.5
6	*Lecanicillium* sp. BV6	0
7	*Fomitopsis* sp. BV7	0
8	*Clitopilus* sp. BV8	7,943.1 ± 0.4
9	*Tyromyces* sp. BV9	232.6 ± 0.4
10	*Mycena* sp. BV10	0
11	*Xylaria* sp. BV11	0
12	*Trametes* sp. BV12	0
13	*Ganoderma* sp. BV13	40.6 ± 0.2
14	*Hexagonia* sp. BV14	0
15	*Tyromyces* sp. BV15	0
16	*Coriolus* sp. BV16	126.3 ± 0.1
17	*Nemania* sp. BV17	564.2 ± 0.3
18	*Nigrospora* sp. BV18	252.1 ± 0.6
19	*Inonotus* sp. BV19	0
20	*Psathyrella* sp. BV20	0
21	*Mycena* sp. BV21	0

3.2. Optimal conditions for the production of laccase by the fungus *Clitopilus* sp. BV8

Effect of carbon and nitrogen sources:

The strain *Clitopilus* sp. BV8 was cultured on a medium supplemented with carbon and nitrogen sources from 0 to 9 days of fermentation at 30°C, pH 6.5. The ability to produce laccase enzymes was significantly affected by different carbon sources. The highest enzyme activity was 46 U/mL in the medium supplemented with tomato substrate and the lowest was 18 U/mL in the medium supplemented with corn substrate after 7 days (Figure 1A).

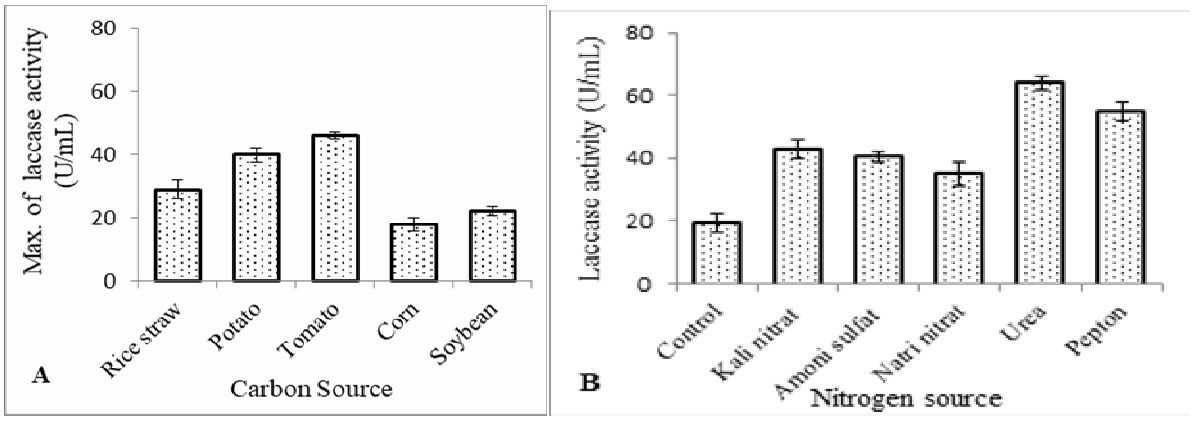

Figure 1. Effect of carbon source (A) and nitrogen source (B) on laccase biosynthesis by *Clitopilus* sp. BV8

The results of figure 1B showed the biosynthesis of laccase was higher than that of the control samples on the media containing nitrogen sources. When a nitrogen source was added to the medium, the laccase activity was between 1.8 and 3.4 times higher than in the control sample. The nitrogen source from urea that biosynthesized the enzyme was highest at 64 U/mL and the lowest from sodium nitrate was 35 U/ml after 9 days of fermentation. Lam and Chien (2013) demonstrated the influence of carbon sources on the growth and development of fungal strains. The fungus *Trametes maxima* CPB30 was surveyed on 8 carbon sources and found that the laccase activity in the medium containing galactose was the lowest at 18 U/ml. In cultures containing other carbon sources, the fungus grew quite well, however, PDA medium was suitable for laccase biosynthesis (579 U/mL) [17]. According to Mai *et al.* (2012), the nitrogen source used 3 g peptone and 1.5 g peptone combined with 1.5 g $(NH7_4)_2SO_4$ for high enzyme activity from 101 U/mL to 120.5 U/ml. However, when there is a combination of inorganic nitrogen and organic nitrogen sources, which can change the pH of the medium, the synthesis of laccase enzyme is higher. The addition of nitrogen source affects the culture medium, and adjusts the pH of the culture medium to facilitate the synthesis of laccase enzyme [16]. Thus, carbon sources from tomatoes and nitrogen from urea increased the biosynthesis of laccase after 7 days of fermentation of *Clitopilus* sp. BV8.

3.3. Effect of temperature and pH

The strain *Clitopilus* sp. BV8 produced the highest laccase enzyme at 25°C (69 U/mL) and the lowest at 40°C (16 U/mL). When the temperature increases from 20 to 25°C, the ability of the fungus to produce enzymes also increases. However, when the temperature increased above 30°C, the fungus did not grow well, so the enzyme activity decreased (16 U/mL) (Figure 2A).

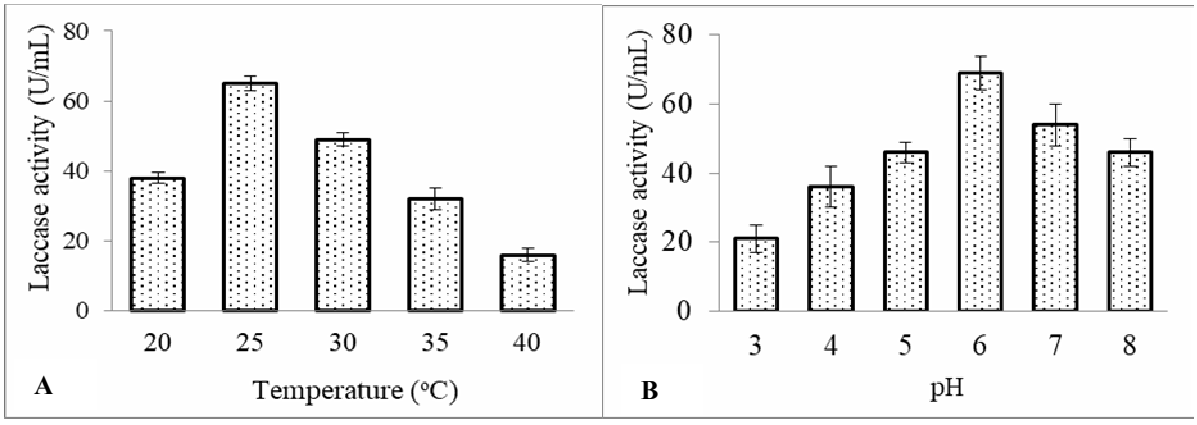

Figure 2. Effect of temperature (A) and pH (B) on laccase biosynthesis by *Clitopilus* sp. BV8

The strain *Clitopilus* sp. BV8 can grow and develop in the pH range from 5.0 to 8.0. The highest laccase activity reached 69.4 U/mL at pH 6.0 and decreased slightly when the pH increased to 7.0 and 8.0. At pH 3, the laccase biosynthetic activity from the fungal strain reached the lowest value of 21.1 U/mL after 9 days. Each fungal species has an appropriate pH range for the best growth and enzyme biosynthesis (Figure 2B). Some studies related to this direction such as Hang *et al.* (2017) studied strain FBV60 (*Pycnoporus* sp. FBV60.) isolated in Ba Vi. Based on morphology and gene sequence, the studied fungus is classified into the phylum Basidiomycetes, genus *Pycnoporus*. The results showed that the fungal strain grew and developed well in the pH range from 4.0 to 9.0, but at pH 6.0, the highest laccase activity was synthesized at 20.47 U/L after 8 days of culture. At pH 3.0 and 10.0, the fungus almost did not grow, the biomass was low and did not produce laccase [18].

3.4. Characterization of laccase from *Clitopilus* sp. BV8 (*Clito*Lac)

The crude enzyme solution obtained by liquid fermentation was further precipitated with various solvents, including ethanol and the inorganic salt ammonium sulfate (40, 50, 55, 60, 65, and 70%). Precipitation was carried out at 18°C, and centrifuged (10 min, 4,000 rpm) to collect protein residue. The fractions obtained after centrifugation and solvent evaporation were reconstituted with 100 mM sodium acetate buffer (pH 5.0) to determine enzyme activity. The results are shown in table 2.

Table 2. Precipitation of crude laccase by different methods

Precipitation method	Concentration (%)						
	40	50	55	60	65	70	Laccase activity (U/mL)
Ethanol	43 ± 0.1	56 ± 0.18	98 ± 0.11	67 ± 0.27	59 ± 0.2	40 ± 0.31	
Amoni sulfate	114 ± 0.15	126 ± 0.25	108 ± 0.28	84 ± 0.25	79 ± 0.14	67 ± 0.32	

The results of table 2 showed that the laccase enzyme activity in the ethanol fractions was low compared to the ammonium sulfate fractions. At the ethanol precipitation fractions, with a solvent concentration of 55%, the highest enzyme activity was 98 U/mL, when the concentration was increased above 55%, the enzyme activity decreased to 40 U/mL at 70%. The highest laccase activity was determined in the ammonium sulfate fractions with a solvent concentration of 50% and the enzyme activity was 126 U/mL. When gradually increasing the solvent concentration, the enzyme activity tended to decrease slightly and reached the lowest value at 70% with enzyme activity of 67 U/mL. The enzymatic activity of ammonium sulfate precipitation was 1.28 times higher than that of ethanol precipitation. Therefore, the method of enzyme recovery by ammonium sulfate inorganic salt is chosen because this method has less effect on laccase activity compared with polar solvent ethanol.

The pH value of laccase was determined between 3.0 and 8.0. The results showed that the laccase enzyme activity obtained from the strain *Clitopilus* sp. BV8 reached a relatively high value in the pH range from 5.0 to 6.0 and reached the highest value of 103 U/mL at pH 5.0. When the pH increased to 6, the enzyme activity decreased slightly by 6 U/mL compared to the enzyme activity at pH 5. After the pH continued to increase, the enzyme activity started to decrease sharply and only reached 55 U/ml at pH 8 (Figure 3A).

Optimal temperature has a great influence on the activity and rate of the reaction, each enzyme only works at a suitable temperature limit. The optimum temperature of the reaction between the enzyme laccase obtained from *Clitopilus* sp. BV8 with ABTS substrate was carried out in the temperature range from 30 to 60°C. The results

showed that the enzyme activity increased gradually from 100 U/ml at 30°C to the highest enzyme activity at 40°C reaching a value of 124 U/mL, and started to decrease gradually after 40°C. After increasing the temperature, the enzyme activity gradually decreased to 17 U/ml at 60°C (Figure 3B).

Thus, the research results showed that laccase biosynthesized from the fungus *Clitopilus* sp. BV8 has an optimum temperature of 40°C and pH 5.

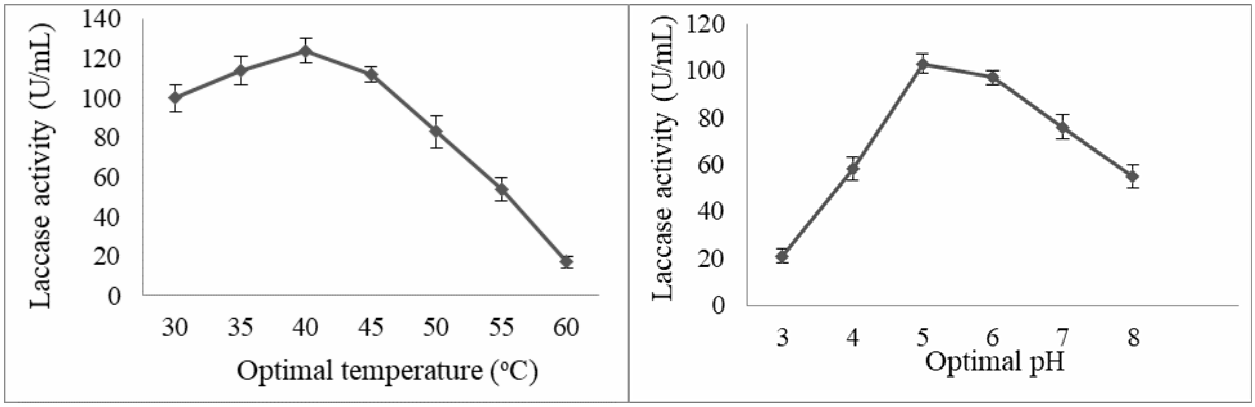

Figure 3. Optimal temperature (A) and pH (B) of *Clito*Lac

3.5. Effect of *Clito*Lac-assisted extraction time on total polyphenol content

The hydrolysis time strongly affects the extraction process of green tea leaves. The longer the extraction time, the higher the efficiency. However, up to a certain point, the extension of extraction time will not be effective. The results of the investigation of the combined hydrolysis time between ultrasound and enzyme on the extraction yield and the main compounds in the extract are shown in figure 4.

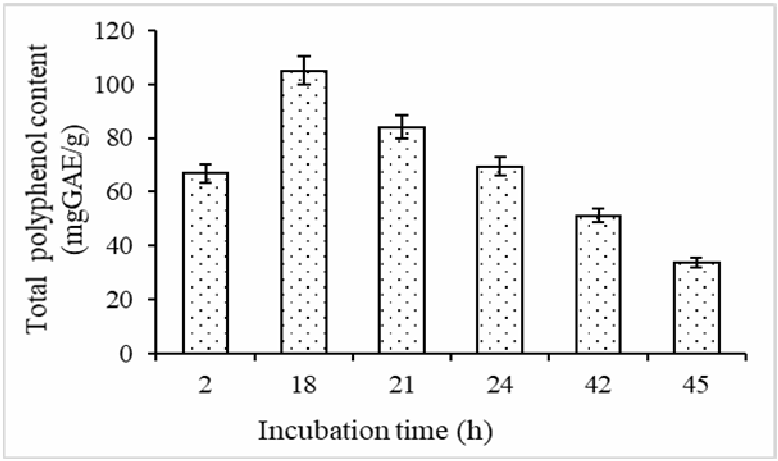

Figure 4. Effect of time on total polyphenols content of tea leaf extract

When increasing the hydrolysis time from 2 hours to 18 hours, the polyphenol content increased rapidly from 66.85 mgGAE/g to 105.04 mgGAE/g and 18 hours is the extraction time to obtain the highest concentration. Continuing to increase the extraction time to 21 hours, the polyphenol content began to decrease, reaching a value of 84.07 mgGAE/g. At 24 hours, the polyphenol content reached 69.49 mgGAE/g. When increasing the time to 42 hours and 45 hours, the polyphenol content decreased sharply reaching 51.29 mgGAE/g and 33.79 mgGAE/g, respectively. It was found that the polyphenol content reached the lowest value at 45 hours and had a high difference compared with the extraction time of 18 hours, reaching a value of 71.25 mgGAE/g. The above results are similar to the study of Thuy *et al.* (2020)[19] on soursop

leaves. If the extraction time is short, the amount of biologically active substances are not completely extracted, but if the time is too long, the active ingredients will be oxidized, and the quantity of the active ingredients will decrease. The survey results showed that the polyphenol content reached the highest level of 94.541 mgGAE/g in the extraction time of 30 minutes. However, quite a lot of reduction between 30 - 45 minutes of extraction [19]. In another study by Quyen *et al.* (2016) on soil gourd leaves. When increasing the extraction time from 10 to 40 minutes, the content increased rapidly from 17.16 to 34.80 mgGAE/g extract and 40 minutes was the time to obtain the highest polyphenol content. However, if the extraction time was increased to 50 and 60 minutes, the polyphenol content increased only slightly and there was almost no difference [20].

3.6. Effect of *Clito*Lac-assisted extraction time on antioxidant activity

The study results show that, the correlation between total polyphenols content for the antioxidant capacity of tea leaf extract. The DPPH free radical technique is frequently used to test antioxidant activity in a very short amount of time when compared to other methods. Several chemical techniques have been investigated to show the strong scavenging potential of tea leaf extract dried extracts. These technologies, however, are not inexpensive and produce a huge number of pollutants. The hydrolysis product of tea leaf extract produced by enzyme-assisted extraction was tested for free radical scavenging activity using DPPH as a free radical in this research [21].

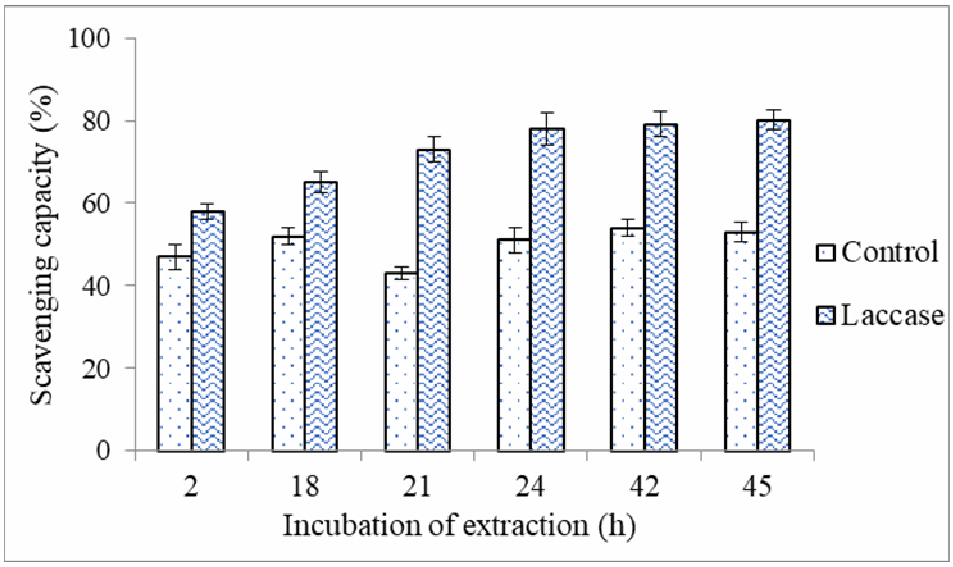

Figure 5. Effect of incubation of extraction on scavenging capacity: (▨) Enzyme-assisted extraction (10U/gds) and (□) inactivated enzyme (control)

Figure 5 shows that, the capacity of dried extract from tea leaf extract for scavenging capacity of free radical. A suitable for total polyphenols content, the hydrolysis products obtained after treatment with laccase showed that, the highest scavenging capacity at 24 hours of incubation (78.23%). A negligible difference was observed with hydrolysis products obtained from 24 hours to 42 hours with scavenging capacity from 79.13 to 80.21%, which were higher than the control (inactivated enzyme) with scavenging capacity from 47.76 to 53.54%.

3.7. Effect of *Clito*Lac enzyme activity on total polyphenol content

The results obtained in figure 3.10 show that enzyme activity has a great influence on polyphenol content. When enzyme activity increased from 1 U/gds to 2 U/gds, the polyphenol content increased sharply from 46.71 mgGAE/g to 90.74 mgGAE/g. However, enzyme activity increased from 3 to 5 U/gds, the polyphenol content increased slightly from 115.32 mgGAE/g to 136.15 mgGAE/g.

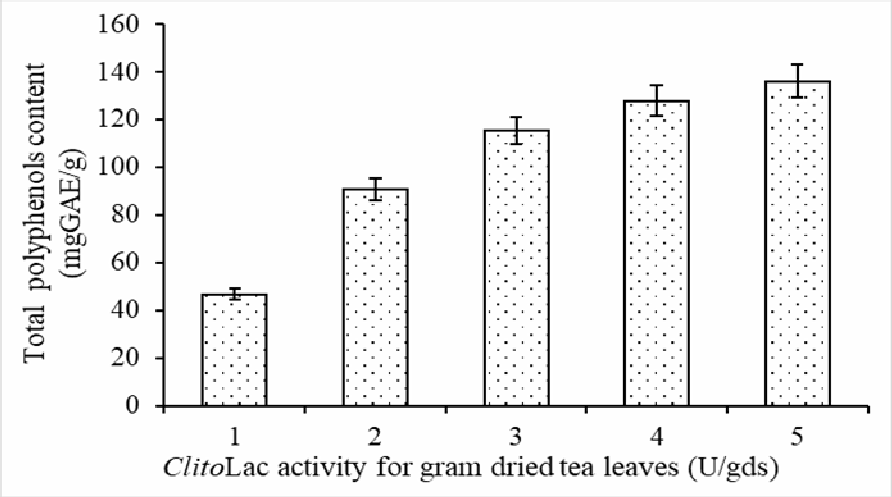

Figure 6. Effect of *Clito*Lac enzyme activity on total polyphenols content of tea leaf extract

It was found that the polyphenol content at the highest value (5.0 U/gds) and the lowest value (1.0 U/gds) had a big difference. This can be explained because when the substrate and enzyme content increase, the reaction rate increases. However, when the enzyme concentration was saturated with the substrate concentration, the reaction rate remained unchanged or increased with increasing enzyme content. So, using laccase enzyme with increasing activity significantly improved the release of products. For that reason, laccase catalyzes the hydrolysis of glycosidic bonds of two or more carbohydrates. Therefore, this enzyme plays a role in the degradation of lignocellulose in plant cell walls. When cells are broken down, it is released from the components of the cell, including proteins, which are known to be complex with polyphenol compounds. Thus, laccase enzyme at 3-5 U/gds was suitable under the above condition. In a study by Dung and Huong (2019) on custard-apple shells using celluclast enzyme. The results showed that, when increasing the enzyme ratio from 0.5% to 1.5%, the polyphenol content increased from 35.89 mgGAE/g to 47.66 mgGAE/g. However, when increasing the enzyme rate to 2.5%, the polyphenol content decreased to 42.65 mg GAE/g [22]. In another study by Buu *et al.* (2018) on the extract of black garlic, the content of polyphenol compounds increased with the increase of cellulase enzyme concentration from 0% to 0.06%, respectively. The value of obtained polyphenol compounds increased from 6.52 mgGAE/g to 11.05 mgGAE/g. However, when the cellulase concentration increased to 0.1%, the obtained polyphenol compounds remained unchanged at 11.11 mg GAE/g [23].

3.8. Effect of *Clito*Lac activity on antioxidant activity

The study results show that, the correlation between polyphenol compounds for the antioxidant capacity of tea leaf extract.

As shown in table 3, the effect of different *Clito*Lac activity (from 1.0 to 5.0 U/gds) was investigated. The antioxidant capacity of the initial dried extract (control sample) was 53.67%. Enzyme treatment had a significant effect on the antioxidant capacity of the samples at 1.0 U/gds compared with the control samples (65.56% and 53.67%, respectively). However, as the results, in the rank of *Clito*Lac activity from 2.0 to 3.0 U/gds a significant change in the liberation of products was observed to be 70.35 to 73.29% for scavenging capacity. The scavenging capacity at 5.0 U is much higher than the control (78.05 and 53.67%, respectively). At 4.0 U/gds, leading to significant changes in the products. However, at higher *Clito*Lac (e.g. up to 4.0 U/gds), the reaction products wouldn't be increased significantly. The highest scavenging capacity using *Clito*Lac (5.0 U/gds) in the enzyme-assisted extraction process was up to 78.05% at 50°C, pH 5.0 during 24 hours incubation. In a study by Buu *et al.* (2018) on

subjects studying the extract of black garlic, the results showed that the DPPH free radical scavenging ability increased when the enzyme concentration increased from 0% to 0.1%, the ability to DPPH free radicals increased from 22.75% to 61.08%. However, there was no difference in DPPH free radical scavenging ability obtained at 0.06% enzyme concentrations; 0.08% and 0.1% [23]. Thus, compared with control extraction, the antioxidant yields were higher in enzyme-assisted extraction system.

Table 3. Effect of different *Clito*Lac activities on antioxidant activity

Enzyme activity (*Clito*Lac, U/gds)	Scavenging capacity (%) obtained by enzyme-assisted extraction (40°C, pH 5.0, 24 hours)	
	*Clito*Lac (test sample)	The inactivated enzyme (control sample)
0	-	53.67 ± 0.12
1	65.56 ± 0.12	-
2	70.35 ± 0.10	-
3	73.29 ± 0.18	-
4	77.79 ± 0.17	-
5	78.05 ± 0.15	-

4. CONCLUSION

In this study, 21 fungal strains were screened for laccase activity, strain *Clitopilus* SP. BV8 showed the highest activity at 7,943.1 U/L. Fungal strains were fermented for 7 days on medium containing the following components: $MgSO_4$ (0.5 g/L); KH_2PO_4 (1.5 g/L); glucose (5 g/L); pH 6.0, tomato (2%), urea (2 g/L) and cultured at 25°C under the condition of continuous shaking at 200 rpm, the highest laccase activity was 65 U/mL. Crude laccase enzyme was concentrated from the culture solution with optimum temperature and pH at 40°C and pH 5.0, respectively. Total polyphenol content released and antioxidant activity from green tea leaf extract supported by laccase enzyme were also evaluated. The results showed that in optimal conditions for hydrolysis of tea leaves in 18 hours, the enzyme/substrate ratio was determined to be 5.0 U/gds at pH 5 at 40°C. Total polyphenol content and antioxidant activity reached the highest values of 136.15 mgGAE/g and 78.05%, respectively.

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FIRST RECORDS OF TWO PARASITOIDS OF *Episparis tortuosalis* A SERIOUS PEST OF *Chukrasia tabularis* IN VIETNAM

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ABSTRACT

Chukrasia tabularis A. Juss is a valuable tree species that contributes to the economic significance of Vietnam where it is grown to produce furniture, doors, window frames, veneers, and decorative panels. However, *C. tabularis* plantations in the country are frequently attacked by the leaf defoliator *Episparis tortuosalis*. This study was designed to illustrate the morphological characteristics of *Ganaspis* wasps and *Megaselia* flies, which were found to be natural parasitoids of *E. tortuosalis* in *C. tabularis* plantations. Our field surveys revealed that the percentage of *E. tortuosalis* larvae and pupae parasitized by *Ganaspis* sp. was significantly lower than by *Megaselia* sp. and the parasitism rate of *Ganaspis* sp. varied significantly between Hoa Binh and Nghe An provinces meanwhile the parasitism rate of *Megaselia* sp. did not differ between these two study sites. These findings suggest that *Ganaspis* sp. and *Megaselia* sp. would be the potential biological control agents for effective and sustainable management of *E. tortuosalis* in the future.

Keywords: *Parasitoid, Ganaspis, Megaselia, Episparis tortuosalis, identification, pest, morphology.*

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1. INTRODUCTION

A wide array of insect pests have caused significant impacts on forest trees and forest ecosystems in different ways such as tree mortality, growth and vigor, defoliation and leaf loss, changes in species composition and structure, widespread of tree diseases, economic losses, and ecological consequences [1 - 4]. The severity of infestations varies depending on insect species. For example, bark and wood borers cause extensive damage to the vascular tissues and structural integrity of trees. This damage leads to tree mortality, especially when infestations occur in large numbers or during outbreaks [5]. These borers also selectively target specific tree species, leading to changes in the dominance and distribution of tree species within a forest [6]. As a result, tree mortality disrupts forest ecosystems, affects biodiversity, finally loss of timber and forest resources [7]. In addition, defoliators cause a reduction in the photosynthetic capacity of trees, leading to reduced growth and energy reserves, stunted development, and decreased overall tree vigor [8]. Weakened trees are more susceptible to other stresses, such as drought, diseases, and secondary insect infestations [9].

Effective management of forest tree pests often involves a combination of multiple approaches, including monitoring, early detection, biological control, cultural practices, and targeted pesticide use when necessary [10, 11]. Among these methods, biological control using their natural enemies is a valuable tool in the integrated pest management (IPM) programs aiming to minimize their ecological and economic impacts while promoting sustainable forest management

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[12, 13]. Parasitoids are insects that lay their eggs on or inside another insect host, which the larvae then feed on and eventually kill. They are important natural enemies of many pest insects and are commonly used in biological control programs to manage pest populations [14]. Some wasps and flies are considered parasitoids because their larvae lead to the death of the host [15]. These parasitoid wasps and flies are used as the key component of biological control agents in forest ecosystems because of their contribution to preventing outbreaks, and reduction reliance on chemical pesticides, minimizing the negative impact on human health, non - target organisms, and the environment [16]. Interest in parasitoid - based biological control of insect pests has grown considerably in recent decades and has mostly focused on Bethilidae, Figitidae, Braconidae, Ichneumonidae, Phoridae, and Pteromalidae parasitoids [17, 18]. Many species of parasitic wasps and flies have been extensively studied and utilized for the biological control of forest insect pests such as *Hyssopus pallidus* in *Cydia pomonella* and *Anastatus orientalis* in *Lycorma delicatula* [19].

Episparis tortuosalis (Lepidoptera: Erebidae: Pangraptini) is a main threat to some forest tree species in India, China and Southeast Asia such as *Chukrasia tabularis*, *Michelia champaca*, and *Azadirachta indica*. In Vietnam, one of the recent studies has reported widespread damage from *E. tortuosalis* in *C. tabularis* in plantations, home gardens and streetscapes [20]. The geographical range and the extent of damage caused by this pest have expanded rapidly across seven provinces in four ecological regions of the country between 2013 and 2020 [20]. The control measures of forest insect pests in Vietnam primarily rely on heavy use of chemical insecticides and the long-term frequent use of insecticides may lead to increased insecticide resistance, destruction of natural enemy populations and pest resurgence.

At present, no control measures have been formulated for *E. tortuosalis*. To develop an effective management measure for *E. tortuosalis*, information on its natural enemies is very important. The objectives of the present paper were to identify external morphological characteristics of the parasitoid wasp and fly of *E. tortuosalis*; to evaluate the parasitism rates of these two parasitoid species in each collection province. This study is expected to lay insights into the potential application of natural enemies for the sustainable development of *C. tabularis* plantations in Vietnam.

2. RESEARCH METHODOLOGY

2.1. Collection sites and methods

During 2020 and 2021, one - hundred - last larval instars and one - hundred - pupae of *E. tortuosalis* were randomly collected from two *C. tabularis* plantations in Nghe An and Hoa Binh (50 larvae and 50 pupae from each province). The samples were then taken to the Forest Protection Research Centre (FPRC), Ha Noi where they were reared in the net cages (40 x 60 x 40 cm). The fresh leaves of *C. tabularis* were weekly provided to *E. tortuosalis* larvae as feeding material until pupation and adult moth emergence. When *E. tortuosalis* larvae pupae died, they were placed individually into a 50 mL glass bottle with the top covered by cotton mesh to allow air exchange. Newly emerging adults of parasitoids were collected directly and put into a 1.5 mL Eppendorf before pinning the specimens.

2.2. Identification of parasitoid specimens

Species identifications were undertaken by morphological comparison of specimens with previously published descriptions. Identification of parasitic wasps was based on descriptions by Kasuya *et al.* (2013) [21] and Gonzalez-Cabrera *et al.* (2020) [22]. Identification of parasitic flies was based on description by Disney *et al.* (2014) [23].

Morphological measurements of slide mounted specimens were taken to describe the species using a micrometer mounted in a compound microscope Leica M165C stereozoom microscope (Leica Microsystem, Wetzlar, 54 Germany) with a micrometric scale. The parasitoid wasp and fly images were captured by using a Nikon DS-63 Fi2 camera (Nikon Corporation, Ha Noi, Vietnam) mounted on a Leica M165C

microscope (Leica 64 Microsystems, Wetzlar, Germany). The images were further processed using Adobe Photoshop. Adult voucher specimens were deposited in the insect collection of the FPRC with numbers coded from FPRC111 to FPRC119 for the wasps and FPRC120 to FPRC130 for the flies.

2.3. Data analysis

The number of larvae and pupae dead by parasitoid wasps and flies were observed to calculate the percentage of parasitism (P%) using the following equation.

$$P\% = (n/N) \times 100$$

Where n is the number of parasitized *E. tortuosalis* larvae/pupae; and N is the total number of *E. tortuosalis* larvae and pupae collected.

Independent sample T-tests were performed to test for mean differences in parasitism rate of *Megaselia* sp. and *Ganaspis* sp. larvae and pupae between Hoa Binh and Nghe An provinces at $p < 0.05$ along with a 95% confidence interval. Data were analyzed using GenStat Release 12.1 software package (VSN International Ltd., Hemel Hempstead, UK).

3. RESULTS

3.1. Morphological description of parasitoids

Two kinds of parasitoids were collected from larvae and pupae of *E. tortuosalis* in the laboratory of FPRC. Both of them are currently identified to generic level. Morphological descriptions of them are as below.

Ganaspis sp. (Hymenoptera: Figitidae: Eucoilinae): Body length 2.0 – 2.8 mm. Wing lengths 1.0 – 1.3 mm long. Body color black to dark brown. Leg color is light brown. Sculpture on vertex. Lateral surface of pronotum, mesosoma, and metasoma is entirely smooth. Head round in anterior view, more transverse in lateral view. Pubescence on head sparse glabrous. Sculpture along lateral margin of occiput absent. Gena short. Lateral margin of occiput round. Occiput smooth. Carina expanding from lateral margin of postocciput absent. Anterior ocellus far from posterior ocelli (Figure 1).

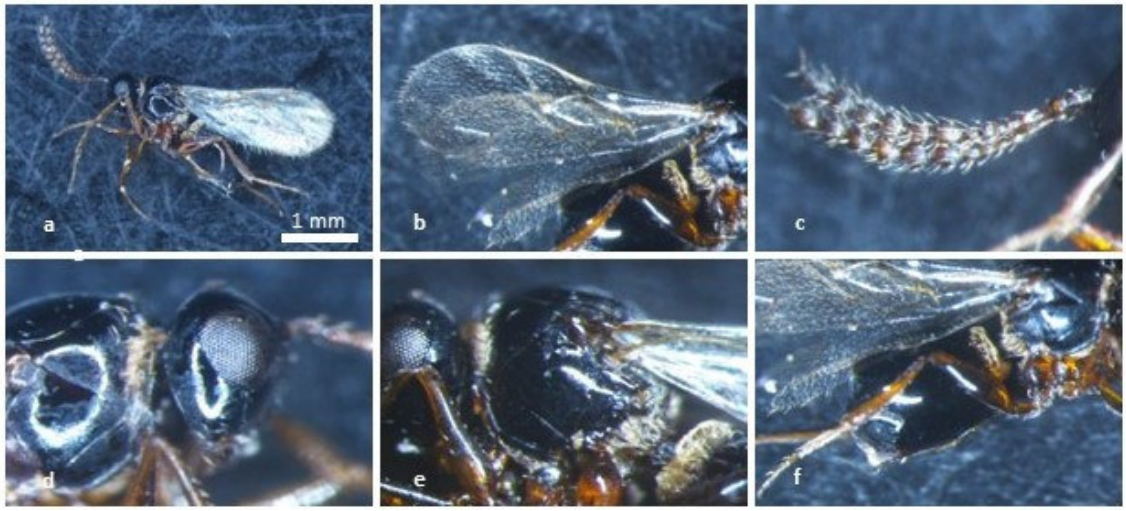

Figure 1. External morphological characteristics of *Megaselia* sp. (a) Lateral view; (b) Forewing and Hindwing; (c) Antenna; (d) Head; (e) Thorax; (f) Abdomen

Megaselia sp. (Diptera: Phoridae): Body length 2.3 - 3.3 mm. Wing lengths 1.1 - 1.6 mm long. Body color black to dark yellow except for brown on the head. Leg color is dark yellow. Costal cilia 0.01 - 0.03 mm long. The outer portion of two axillary bristles 0.04 mm long. Frons dense. Palps dark yellow. Mesopleuron bare. Labrum short. Thorax with two notopleural bristles. Scutellum with an anterior pair of hairs and a posterior pair of bristles. Hypopygium with a pale anal tube. Cercus and proctiger hairs are shorter than epandrium hairs. Lobes of hypandrium vestigial. Dorsal hair palisade of mid tibia extends about 0.6 times its length. Hairs below basal half of hind femur are longer than those of anteroventral row of outer half (Figure 2).

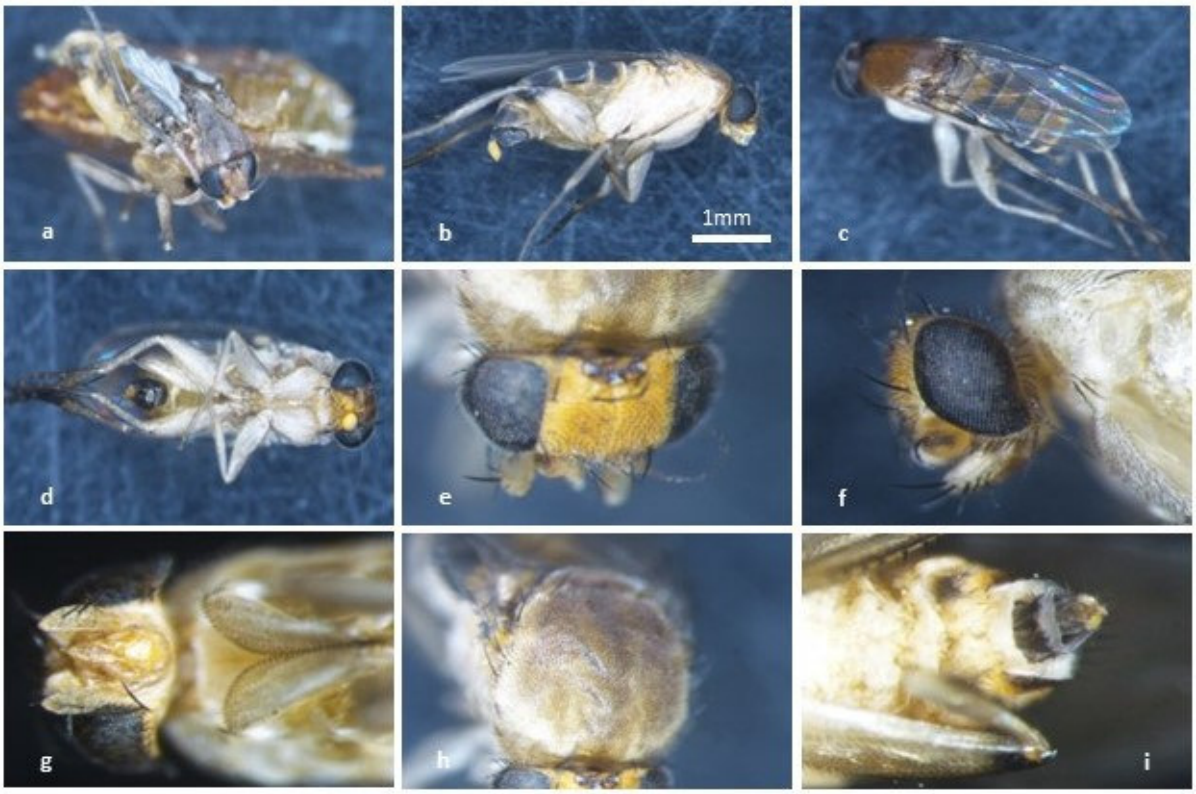

Figure 2. External morphological characteristics of *Megaselia* sp. (a) Newly emerged adult; (b) Lateral view; (c) Dorsal view; (d) Ventral view; (e) Head; (f) Eye; (g) mouth; (h) Thorax; (i) Ventral view of genitalial terminalia

3.2. Parasitism rates of parasitoids

In general, the percentage of larval and pupal *E. tortuosalis* parasitized by *Ganaspis* sp. (5.3 - 7.0% and 6.1 - 8.9%, respectively) was significantly lower than by *Megaselia* sp. (11.7 - 12.1% and 16.8 - 17.5%, respectively) (Table 1).

There was a significant difference ($p < 0.001$) in the percentage of *E. tortuosalis* larvae and pupae parasitized by *Ganaspis* sp. wasps between Hoa Binh and Nghe An. In contrast, this result was not observed in *Megaselia* sp. flies ($P = 0.448$ - 0.521) (Table 1).

Table 1. Parasitism rates of *E. tortuosalis* larvae and pupae caused by *Ganaspis* sp. and *Megaselia* sp. in Hoa Binh and Nghe An provinces

Parasitoid species	Province	Mortality rate (%)	
		Larvae	Pupae
Ganaspis sp.	Hoa Binh	5.3	6.1
	Nghe An	7.0	8.9
P value		*<0.001*	*<0.001*
Megaselia sp.	Hoa Binh	11.7	16.8
	Nghe An	12.1	17.5
P value		*0.448*	*0.521*

4. DISCUSSION

This study is the first that reports two parasitoids of *E. tortuosalis* in *C. tabularis* plantations in Vietnam. *Megaselia* is the largest genus in the family Phoridae (cuttle flies), a member of the order Diptera [24]. There are currently more than 1,400 species of the approximately 4.300 species in this genus have been described so far, and large number of species are found in the tropical and subtropical countries of the world [25, 26]. *Megaselia* genus has high species diversity and richness, and life histories are tremendously diverse despite only a small proportion being known [26]. The genus *Megaselia* exhibits remarkable ecological diversity. Different species within this genus can be found in a wide range of habitats, including forests, grasslands, caves, and human-made structures such as houses, barns and compost heaps. Some species are associated with decaying organic matter, while others are found in association with insects, fungi, or vertebrate carrion. The genus *Ganaspis* is a group of parasitic wasps belonging to the family Figitidae (gall wasps or fig wasps) within the order Hymenoptera [27]. This genus contains numerous species, and taxonomists continue to study and describe new species within this group. Different species within this genus exhibit varying levels of host specificity. Many *Ganaspis* species target specific insect hosts, often associated with galls or plant tissues. Taxonomists continue to study the diversity, biology, and behavior of these *Megaselia* and *Ganaspis* species to better understand the relationships among different species and their interactions with hosts and the environment because this information is crucial for developing effective pest management strategies and conservation efforts.

Researchers and forest owners explore the potential of using parasitoid wasps and flies as biocontrol agents to reduce the reliance on chemical pesticides and promote sustainable pest management practices. Some species of *Ganaspis* wasps and *Megaselia* flies have been reported to parasitize different insects such as *G. brasiliensis* in some frugivorous *Drosophila* species [28 - 30], *Ganaspis* sp. in *Atherigona varia* [31], *M. opacicornis* in *Chrysomela lapponica* [32], *M. scalaris* in *Spodoptera frugiperda*, *Mischocyttarus cassununga*, *Macrotermes gilvus* and honeybee [33 - 35], *M. rufipes* in *Apis mellifera* [36]. Thus, *Ganaspis* sp. and *Megaselia* sp. based biological control agents in this study will hold a great promise as a valuable tool in the development of natural enemies for sustainable management of *E. tortuosalis* in Vietnam. Interestingly, our results found that the percentage that *E. tortuosalis* larvae and pupae parasitized by *Megaselia* sp. flies were significantly higher than those by *Ganaspis* sp. wasps. This finding implies that *Megaselia* sp. might be more important than *Ganaspis* sp. in controlling and killing *E. tortuosalis*.

The expansion programs of *C. tabularis* plantation areas have been strongly promoted and supported by the Ministry of Agriculture and Rural Development in recent years. Therefore, *C. tabularis* is among the most important forest tree species in the country. Increase in defoliation from *E. tortuosalis* presents many challenges for plantation growers [20] since when *C. tabularis* trees are lose its leaves, they can suffer from reduced photosynthesis, which might lead to decreased growth, weakened immune systems, and reduced fruit or seed production. The application of parasitoid wasps and flies in the preventive control of insect pests is limited by several practical issues, particularly rearing and releasing issues [37]. Some species of *Ganaspis* wasps and *Megaselia* flies are commonly used as laboratory model organisms in scientific research because they are relatively easy to rear in the laboratory and their short generation time and large brood sizes make them useful for studying various biological phenomena, such as development, behavior, and genetics. Though parasitoid wasps and flies do not feed directly on artificial diets, a proper artificial diet for mass - rearing their hosts would contribute to increasing the individual populations for mass releases in the field. Indeed, methods for rearing and releasing *Ganaspis* sp. and *Megaselia* sp. have been studied

[38 - 40]. Therefore, further studies are required to focus on biological features, mass rearing, and releasing techniques of *Ganaspis* sp. and *Megaselia* sp. under *C. tabularis* plantation conditions in Vietnam.

5. CONCLUSION

This study provides the first reported data regarding the external morphological identification of two parasitoid species of *E. tortuosalis* including *Megaselia* sp. and *Ganaspis* sp. Although these two parasitoid wasps and flies do not seem to sufficiently reduce pest populations in fields, but this study is the basis for developing them into potential biological control agents. Additional studies are required to clarify unknown aspects of two parasitoids such as taxonomy, biology, and rearing techniques as well as releasing methods of *Megaselia* and *Ganaspis* species and implement biological control as a successful and important component of integrated pest management.

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ENRICHMENT AND RECOVERY OF RESISTANT MALTODEXTRIN FROM RICE STARCH BY ETHANOL PRECIPITATION AND ION - EXCHANGE CHROMATOGRAPHY

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Pham Thi Binh[2], Nguyen Van Loi[3], Nguyen Thi Van Linh[4]

ABSTRACT

Two processes are introduced as approaches for enriching and recovering resistant maltodextrin from rice starch-based pyrodextrin hydrolyzate: Ethanol precipitation and ion-exchange chromatography. For ethanol precipitation technology, the parameters include: Pyrodextrin solution concentration of 40%, rate of ethanol volume versus dry mass of pyrodextrin is 10 times (v/w), and time for separation precipitation of 5 hours. For the ion-exchange chromatography technology, it requires the strong acid cation exchange resins in Ca^{++} form with separation conditions including: Space velocity SV 0.25 - 0.50, initial pyrodextrin solution concentration of 30-40°Bx. The product recovery by ethanol precipitation provides a high separation yield, while the ion-exchange column yields less at higher purity. Both technologies can be considered applicable in practice.

Keywords: *Ethanol precipitation, ion exchange, pyrodextrin, resistant maltodextrin.*

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1. INTRODUCTION

Resistant starch as an excellent dietary fiber is an important part of the human diet and has been considered as an essential nutrient to humans, especially for obesity, diabetes, and cardiovascular diseases [1, 2]. Pyrolysis and enzymatic hydrolysis have been recognized as two principal steps in the process of producing a new type of soluble dietary fibers called resistant maltodextrins (RMD) [3, 4]. The pyrolysis is the first step and its product is pyrodextrin, which is used as the starting material for RMD production through intermediate stages of hydrolysis, purification, enrichment by separation, and drying [5, 6, 7]. The hydrolyzed product after saccharification and purification (filtration, decolorization, and demineralization) is a mixture of sugars, and oligosaccharides (dextrin, maltodextrin) with different molecular sizes or polymerization degrees (DP). According to previous research findings, the indigestible fraction (IDF) of this mixture is approximately 65-75%, the total dietary fiber content (TDF) is approximately 55 - 65%, and the DE is approximately 45 - 55% [8]. To produce a product with a higher level of IDF, TDF, as well as with a maltodextrin DE (≤ 20), it is necessary to remove sugars with polymerization degrees of DP1 and DP2, leaving only the components with DP > 3 [9, 10].

Two commonly used methods for sugar separation are ethanol precipitation and ion exchange chromatography. Precipitation is considered to be a simple unit operation. It is widely used to concentrate biomolecules, mainly proteins and sugars, in aqueous solutions [10, 11, 12]. Specifically, precipitation with ethanol has low capital costs and low operating costs. The precipitating agent can be recycled by simple distillation after the liquid and precipitated phases have separated, thereby reducing the

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environmental impact of the effluent. The ethanol fractionation method is based on the different solubility of oligosaccharides in ethanol solutions with different concentrations [13, 14, 15]. When performing chromatographic separation, the large sugar molecules will exit first, the double (DP2) and single sugars (DP1) will come out last. Therefore, for pyrodextrin solution mixtures, maltodextrin and dextrin will be recovered first, dextrose will be collected last [16 - 19].

The aim of this study was to determine the technological parameters for enriching and recovering rice starch-based resistant maltodextrin by removing low-molecular-weight sugar components in order to evaluate their industrial-scale applicability.

2. MATERIALS AND METHODS

2.1. Materials

Rice starch of the IR50404 variety was purchased from Hoa Phat Food Processing & Trading Company Limited (HCMC, Vietnam). After purification and drying at 105°C, the rice starch containing 93.9% carbohydrate, 1.03% protein, 0.22% lipid, and 5% moisture was used as a native starch sample. Acetic acid ($C_2H_4O_2$), NaOH and HCl, were purchased from Sigma-Aldrich Corp. The high purity α-amylase from *Bacillus licheniformis*, protease from *B. licheniformis*, amyloglucosidase from *Aspergillus niger*, total dietary fiber (TDF) kit K-TDFR-100A/KTDFR-200A 04/17, and K-GLUC (GOPOD Format) were purchased from Novozymes (Denmark) and used as received. Resin Lanlang® CS-1 is a strong acid cation resin in calcium form specified for glucose and fructose separation was manufactured and provided by Taiyuan Lanlang Technology Industry Corp (China RPD). Resin Purolite® PCR642K in polystyrene gel form is a strong acid cation resin in potassium form purchased by Purolite Ltd. (UK). Ambersep® 200 H also is a strong acid cation resin having sulphonic acid located in polystyrene matrix. This resin is manufactured by Rohm & Haas (USA) and provided by Megazymes Ltd. (Ireland).

2.2. Pyrodextrin preparation and enzymatic hydrolysis

The overall process of resistant pyrodextrin production was explained as follows: Six kilograms of rice starch (3 - 5% moisture) were put into a rotary mixer (Vietnam), 600 ml of 1% HCl solution was sprayed with compressed air while rotating the mixer, and after being uniformized through an overnight equilibration at room temperature, the sample was dried at 105°C in a rotary roaster (Vietnam) for 6 hours until its moisture content was 3 - 5%. The dextrinization process was then carried out in the same equipment by raising the temperature to 170°C for approximately 90 minutes. The counting of the heating time started when the sample temperature reached the target temperature, and the heating stopped when the whiteness of the starch reached 65 - 67. The pyrodextrin was hydrolyzed by two steps with two enzymes for liquefaction and saccharification. Deionized water was added to 5 kilograms of pyrodextrin, the substrate concentration was 33 - 40%, and pH 5.8 was adjusted with 20% sodium hydroxide before adding 0.4% by weight of a solution of -amylase (Termamyl SC, Novozymes A/S, Denmark, activity 2860 IU/mL) to liquefy at 95-97°C for 60 minutes.. The saccharification was performed using amyloglucosidase (AMG 300 L, Novozymes, Denmark, activity 518 U/mL) at a concentration of 0.2%, pH 4.5, and temperature of 60°C for 5 hours. Most of the solution was refined through conventional processes such as decoloring and filtration with activated charcoal and deionization with ion exchange resin. Clear liquid products after liquefaction, saccharification, and purification had an index of dextrose equivalent (DE) of 54 - 57, a soluble solid concentration (Brix degree) of 29 - 31°Bx, a content of indigestible fraction (IDF) of 64 - 67% (db), a protein content < 0.6% (db), a lipid content < 0.04% (db), and a color of 65 - 70 [8].

2.3. Ethanol precipitation of hydrolyzed pyrodextrin solution

The ethanol precipitation was done following Woo and Moon with minor modifications [10]. Take 25 mL of pyrodextrin hydrolysate and add

96% ethanol by volume fractions of 5, 7, 10, and 15 times the mass of soluble solids in the dextrin solution (12 g). Stir well and then leave the mixture at laboratory conditions for 4 hours to form a precipitate. Remove all the liquid from the top by decanting to another beaker. Use warm water at 60°C to redissolve the precipitate and then vacuum concentrate to remove excess ethanol. Adjust the volume to 25 mL exactly equal to the original volume. Measure the concentration in °Bx to calculate the recovery yield (rate of precipitated fraction) and determine the level of indigestibility. Precipitation incubations of 1, 3, 5, 10 and 15 hours were used to determine the appropriate precipitation time. To determine the appropriate hydrolysate concentration, pyrodextrin solutions with 20, 30, 40 and 50% (°Bx) were tested. The recovery of maltodextrin by ethanol precipitation at the scale of 5 l each batch was carried out in a 30 liters device with parameters derived from a small-scale test.

2.4. Ion-Exchange chromatography for separation

The column is made of SU304 stainless steel with 2 shells, 46 mm inner diameter, 1009 mm long. Hot water supply system was provided by a thermostat (JSRC-13C, JS Research, Korea) and a built-in pump in the device. Deionized water for column washing, elution was supplied with the Lead Fluid BT102S pump with rpm or mL/min indication. The system consists of 3 columns that can be operated independently or connected by valves and heat-resistant plastic pipes. The first column uses Lanlang® CS-1 resin which is a strong acid cation exchange resin in the form of Ca^{++}, the second column is filled with Purolite® PCR642K resin in the form cation K^+ and the third column uses Ambersep® 200 H resin which also is a strong acid cation exchanger in the H^+ form. The column was filled with resin to a height of about 90 cm (measured after column washing). The volume of filled resin was about 1,500 ml. A pyrodextrin solution of 40% concentration was injected into the column at a flow rate of 0.5 bed volume (BV) (equal to space velocity SV = 0.5), i.e. 0.75 l/hours or 12.5 ml/min. After finishing loading the sugar solution into the column, use deionized distilled water at 60°C to elute. The fractionation was maintained at a flow rate of 13 ml/min, maintained for 90 min. Sampling every 5 minutes, for a total of 18 times in 90 minutes. The analytical parameters include: Dextrose equivalent (DE), indigestible fraction content (IDF), concentration of dextrose sugar (DP1), maltose sugar (DP2). Expressed in terms of C_i/C_0 where i is the type of component *i* and C_0 is the initial concentration of component *i* in the sugar solution.

Determination of space velocity (SP) by filling 3 columns with the same resin which has been determined from the above-mentioned experiment. Maintain the same condition of 3 columns, only the difference in fluid flow rate is 0.25; 0.50 and 0.75 BV respectively 6.25 mL/min; 12.5 mL/min and 18.75 mL/min. Determine the column feedstock concentration with the difference in soluble solids concentrations of the solution: 30, 40 and 50°Bx. Flow rate 0.5 BV, sample collection every 5 min, and sample aliquots were stored and analyzed in the same manner as described above.

2.5. Indigestible fraction (IDF) and total dietary fiber (TDF)

The indigestible fraction (IDF) contents of the treated starch samples were determined according to the AOAC Official Method 2001.03 with a slight modification [20, 21]. This method was described in our previous study [6, 7, 8]. The TDF content of pyrodextrin or starch was determined according to the AOAC method 991.43 (AOAC, 2003) with some improvements at the HPLC sugar determination stage [22]. Accordingly, the K-MASUG kit (Novozymes, Denmark) was used to determine the total sugars DP1 and DP2 (3 types of sugars: Maltose, sucrose and glucose).

2.6. Physicochemical properties

The pH value of starch in the mixture was determined by taking 25 g of starch (or pyrodextrin) and mixing it with 50 ml of deionized water. Determination of dextrose equivalent (DE) was according to Vietnam National Standard TCVN 10376: 2014 on "Starch Hydrolysis Products - Determination of Reducing Power and Dextrose

Equivalent - Lane and Eynon Constant Title Method". Measure the whiteness of starch or pyrodextrin directly with the Kett C-300 whiteness meter (Kett Electric Laboratory) which has a range of 0 to 100 with 0 representing the darkest, 100 representing the whitest. To measure liquid color, first prepare a pyrodextrin solution with 10% dry matter, i.e., 10°Bx. Then filter through filter paper and then measure optical density at 420 nm and 720 nm using quartz cuvettes 1 cm thick. The difference between two ODs (absolute value) multiplied by 10 is considered as the color of the pyrodextrin [3, 4].

2.7. Statistical analysis

One-way analysis of variance was performed with Minitab software version 16. The least significant differences for comparison of means were computed at $p < 0.05$. All analyses were performed at least in triplicate, and the experimental results were expressed as mean ± standard deviation.

3. RESULTS AND DISCUSSION

3.1. Determination of separation parameters by ethanol precipitation

3.1.1. Ethanol concentration

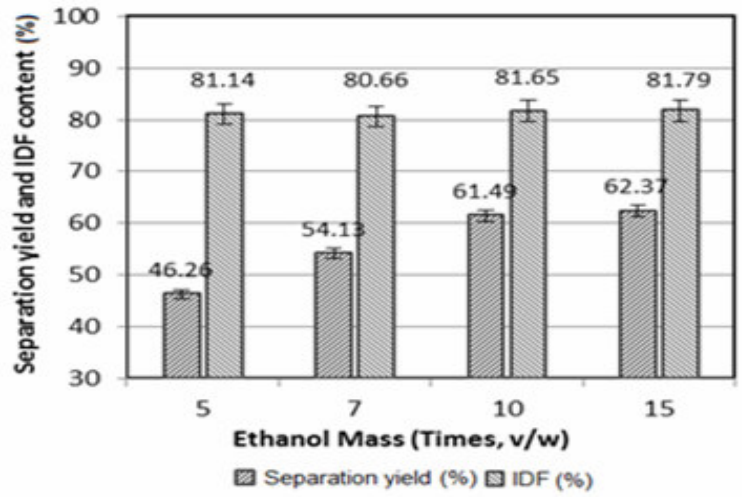

Figure 1. Change in the separation yield and indigestible fraction content (IDF) depends on ethanol concentration

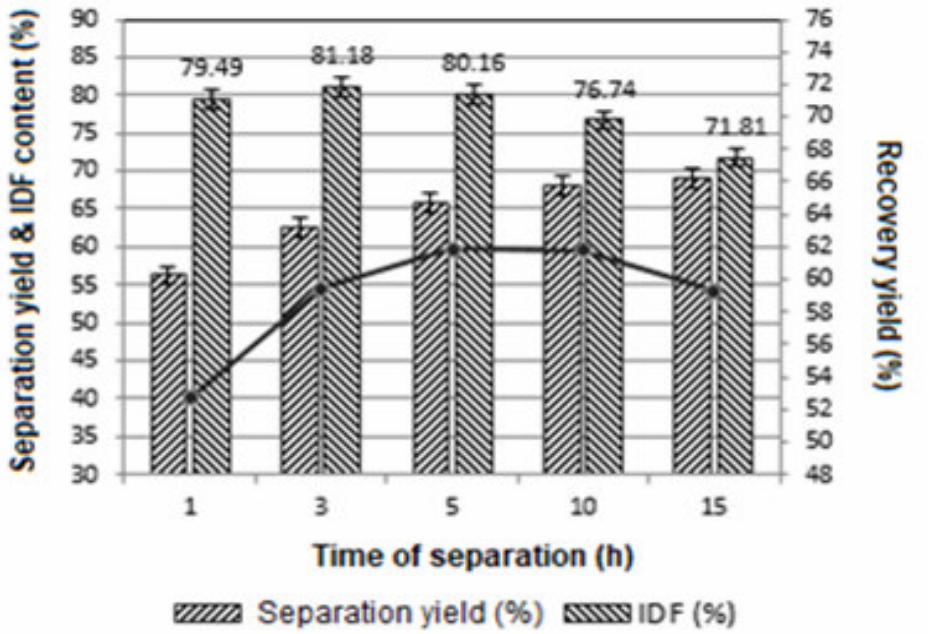

Figure 2. Change in separation yield and indigestible fraction content (IDF) depends on precipitation time

The appropriate ethanol concentration can be determined using two criteria: Separation yield and indigestible fraction (IDF) content. The separation yield was expressed as a percentage of the ratio of the soluble solid mass of the solution after precipitation to that before separation. The initial hydrolyzed pyrodextrin solution had a concentration of 30°Bx, and the ethanol concentration was 96 degrees in all experimental formulas. The results of the determination of separation yield and IDF content of resistant maltodextrin with different ethanol ratios are shown in figure 1. The data show that separation yield increases with increasing ethanol ratios. The highest separation yield was obtained at a 10 : 1 ratio and did not increase significantly at 15 : 1. The contents of IDF in the formulations were all very high and did not differ significantly. From this result, the recommended ethanol ratio is 10:1. Results of this study are completely consistent with many other published results because, as is known, ethanol precipitation is one of the most widely used methods for preparing polysaccharides and sugars, in which ethanol concentration significantly affects the precipitate yield and is usually set at high concentration of 70 - 80% [10 - 15].

3.1.2. Separation time

The separation yield of resistant maltodextrin was determined at various time intervals ranging from 1 to 15 hours. The precipitation parameters were the concentration of solute (30°Bx), ethanol (96 degrees), and the ratio 10 : 1. The results are shown in figure 2. The data shows that the separation efficiency at 1 hours of precipitation is the lowest (56.34%), increasing to 62.48% and 65.70% upon precipitation for 3 and 5 hours, respectively. When the precipitation time was increased to 10 and 15 hours, the separation yield increased significantly, gaining 68.13% and 69.11%, respectively. As a result, the separation yield increases with precipitation time and reaches a maximum after 10 hours. The difference between 10 hours and 15 hours was negligible. In contrast to the separation yield, the content of IDF decreased gradually from 3 hours to 15 hours of precipitation, which decreased markedly after 10 hours. The IDF recovery yield is the separation yield multiplied by IDF, so the value corresponding to each precipitation time is also shown in figure 2. The results show that the recovery yield peaked at 5 hours of separation. Based on these findings, the optimal time to separate the resistant maltodextrin was 5 hours. If the precipitation time is increased, although there is an increase in the separation yield, the product is not beneficial in terms of the recovery yield on the indigestible fraction.

3.1.3. Concentration of hydrolyzed pyrodextrin solution

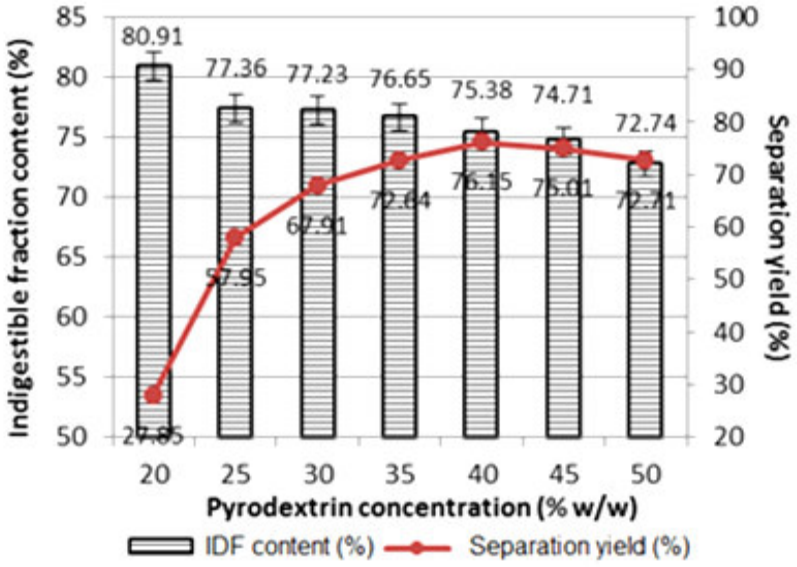

Figure 3. Change in the separation yield and indigestible fraction content depends on concentration of pyrodextrin solution

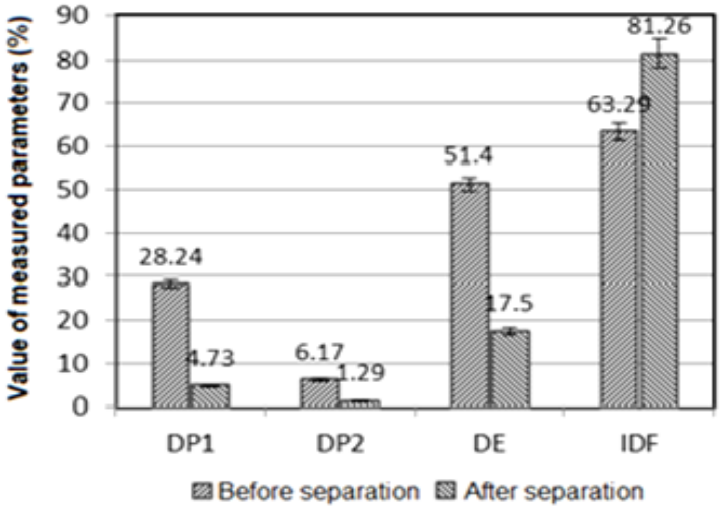

Figure 4. Content of glucose (DP1) and maltose (DP2), DE, and IDF of pyrodextrin solution before and after the ethanol separation

The results of the determination of separation yield for precipitated pyrodextrin solutions with different soluble solid concentrations are presented in figure 3. The precipitation parameters used were ethanol 96 degrees with a ratio of 10 : 1 (v/w) and a precipitation separation time of 5 hours. The data show that separation yield increases with concentration (°Bx), specifically increasing in the range of 20 - 40°Bx, reaching the maximum value at 40°Bx. However, separation yield tends to decrease gradually at 45 - 50°Bx. The IDF contents were all high and decreased gradually with the pyrodextrin concentration. The IDF recovery yield at 40 and 45°Bx was the highest. From this result, the most suitable pyrodextrin concentration for maltodextrin separation is 40 - 45% (in Brix degrees).

3.1.4. Maltodextrin separation by ethanol precipitation on semi-pilot scale

The purpose of this experiment is to verify the sugar separation parameters obtained from three small-scale laboratory experiments. The process of sugar separation was at the scale of 5000 mL of initial solution having 40°Bx, the ethanol ratio was 10 times v/w (20 liters of ethanol at 96°), and the precipitation time was 5 hours. In order to compare the important components of the solution before and after the sugar separation by precipitation method, some parameters such as glucose concentration (DP1), maltose (DP2), indigestible content (IDF) and dextrose equivalent (DE) were determined, and the results are shown in figure 4. The data show that the purified hydrolysate's DP1 (glucose) decreased from 28.24% to 4.73% and the DP2 (maltose) decreased from 6 to 17% to 1.29%. Because DP1 and DP2 were mostly removed from the precipitated solution, there were main components with a polymerization degree of DP3 (maltotetraose) or higher in the precipitate. For this result, the DE value of 51.4 was down to 17.5 after separation. The most interesting effect is the increase in IDF content, which increased from 63.29% before separation to 81.26% in the product after separation. As a result, the ethanol precipitation technique can be used to enrich resistant maltodextrin by increasing the IDF of pyrodextrin solution.

3.2. Determination of separation parameters by ion exchange chromatography

3.2.1. Selection of the ion-exchange resin

The results shown in figure 5 indicate that the strong acid cation exchange resin in the form of Ca^{++} (Lanlang CS-1) gives a lower concentration of DP1 and DP2 after separation than the other two resins, while owning a much higher concentration of high molecular weight sugars (95.36% versus 91.59% and 87.81%). The results in figure 6 also show the advantage of Lanlang CS-1 resin because this resin had the ability to obtain significantly higher content of IDF than the other two resins.

Among the 3 resins, the separation efficiency was in the following order: Lanlang CS-1 > Purolite PCR642K > Ambersep 200H. Therefore, Lanlang CS-1 resin produced and supplied by Lanlang Company was recommended to be used for further studies. However, Purolite PCR642K resin can also be used as an alternative if it has other advantages such as supply or durability.

The resin in Ca^{++} (alkaline earth metal) and K^{+} (alkaline metal) forms have also been proposed to be used for the purpose of sugar (fructose/glucose) separation in a number of foreign publications [16, 17]. However, a comparative study, especially compared with both H^{+}, has not been published at home or abroad [18, 19].

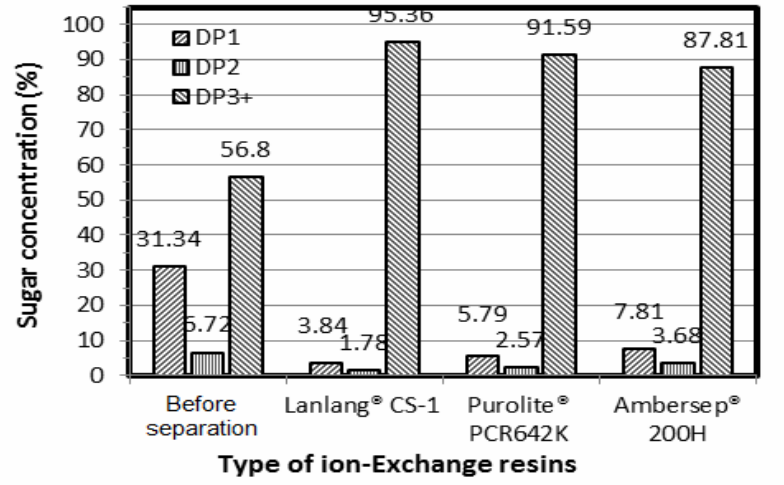

Figure 5. Comparison of concentration of sugar composition between 3 products from ion exchange chromatographic separation using 3 types of resin (40°Bx, S.V. 0,5)

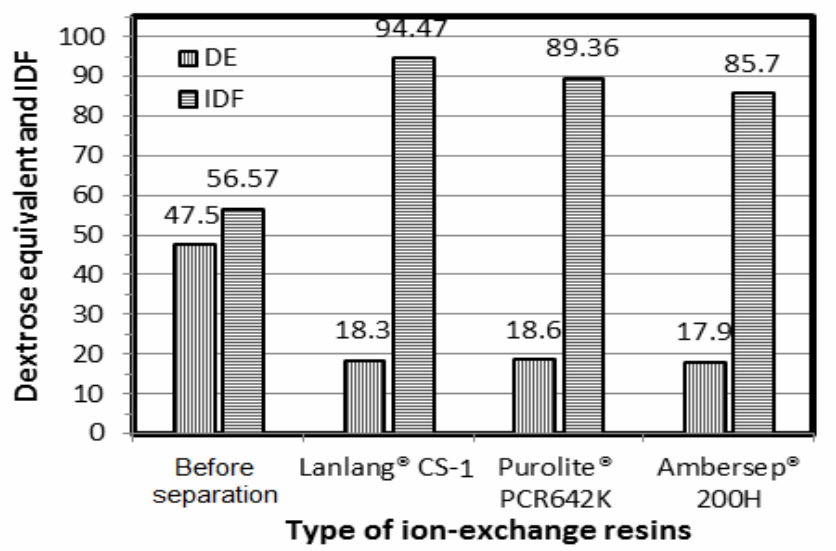

Figure 6. Comparison of indigestible content and dextrose equivalent (DE) between 3 products from ion exchange chromatographic separation using 3 types of resin (40°Bx, S.V. 0,5)

3.2.2. Determination of appropriate space velocity

In this experiment, a strong acid cation exchange resin in the form of Ca^{++} (Lanlang® CS-1) was used to separate the sugars. Separation conditions were maintained similar to the experiment mentioned in Section 3.2.1, only differing in the space velocity (SV): 0.25, 0.5, and 0.75 BV (bead volume). The analyzed parameters include glucose (DP1), maltose (DP2), and sugars with DP ≥ 3 (DP3+). Values are expressed as C_i/C_o as a percentage, i.e., the value of the C concentration at the i-th sampling time relative to the initial concentration. Only DE is expressed in

absolute value. In principle, it was only necessary to determine the DE value to calculate the fractionation stopping time to collect maltodextrin samples and thereby control the DE of the product after drying. This is based on the requirement that maltodextrin have a DE ≤ 20.

The analysis results shown in figures 7 and 8 correspond to a flow rate or space velocity of 0.25, 0.5, and 0.75 BV. The data from figure 7 show that during the first 50 min, no DP1 sugar was eluted. At 55 minutes, glucose at a low concentration (10%) was determined. Maltose appeared earlier than glucose, as early as 35 minutes, but only in relatively low concentrations because maltose itself in the hydrolyzate is much lower than glucose. The more interesting component is the concentration of sugars with DP3+, which was found to occupy a large amount during the first 50 minutes of separation. These substances make up the indigestible component and the dietary fiber. The overlapping area of the DP3+ sugars with the DP1 and DP2 sugars was smaller than the corresponding area in figure 8, indicating that a low space velocity of 0.25 BV is likely to give a higher purity of DP3+ than that of 0.75. The 0.5 BV flow rate also gives the DP3+ separation as good as 0.25 BV (data not presented).

The DE values were 19.8 after 55 min of separation at SV of 0.25 BV, and DE 18.04 and DE 18.7 after 40 and 25 min of separation at SV 0.5 and SV 0.75, respectively. Thus, a small flow rate (SV 0.25) took longer to complete separation than a high SV, although the separation efficiency or the purity of the DP3+ components was lower due to the mixing of DP1 and DP2 at a larger level. From the above results, the SV 0.5 was proposed to be chosen because of the moderate separation time (40 minutes) and the high purity level.

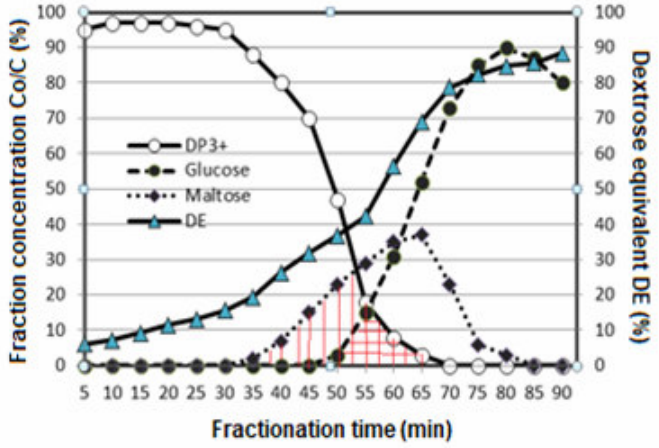

Figure 7. Separation profile of low MW fraction from pyrodextrin hydrolysate of 40°Bx, space verocity of 0.25 BV by Lanlang® CS-1 resin column

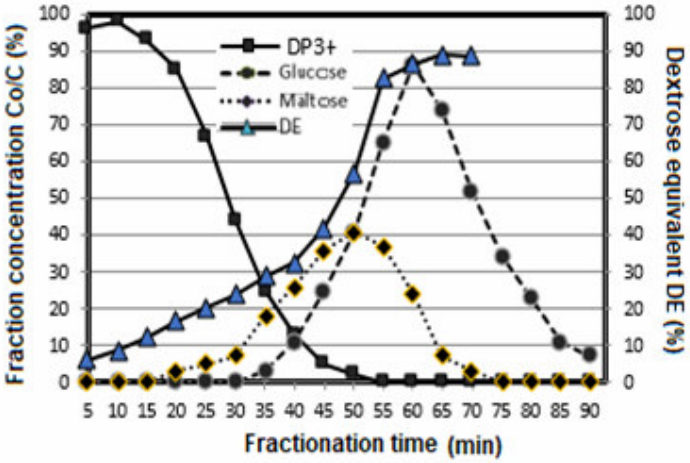

Figure 8. Separation profile of low MW fraction from pyrodextrin hydrolysate of 40°Bx, space verocity of 0.75 BV by Lanlang® CS-1 resin column

3.2.3. Determination of concentration of initial pyrodextrin solution

This experiment maintained the same separation conditions as mentioned in Section 3.2.2. The difference was only in the concentration of the hydrolysate (30, 40, and 50°Bx). The aim was to choose which concentration was suitable for chromatographic separation. In theory, a high concentration is better for separation yield than a low concentration, but there may be some influence effects at high concentrations, such as the need for high temperatures to reduce viscosity. The results of fraction analysis at concentrations of 30°Bx and 50°Bx are shown in figures 9 and 10. The data showed that at the same flow rate (SV 0.5), lower concentrations (30°Bx) gave potential for better separation compared to solutions with high concentrations (50°Bx). This is reflected in the overlap area between the DP3+ concentration curve and the DP1 or DP2 curves. Compromising the benefits of separation capacity or DP3+ enrichment with separation yield, a concentration of 40% (40°Bx) was proposed as the choice. The results of product quality after sugar separation with separation parameters: pyrodextrin hydrolyzate 50°Bx, flow rate 0.5 BV, Lanlang® CS-1 resin, showed that the product quality parameters were satisfactory, such as DE < 20, high IDF content (94.47%), and very low concentration of low molecular weight sugars (3.84% glucose, 1.78% maltose).

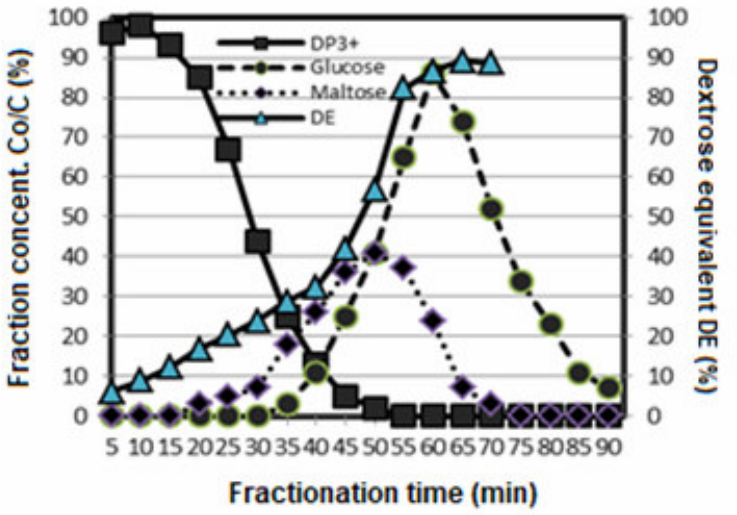

Figure 9. Separation profile of low molecular weight fraction from pyrodextrin hydrolysate of 30°Bx, space verocity of 0.5 BV by Lanlang® CS-1 resin column

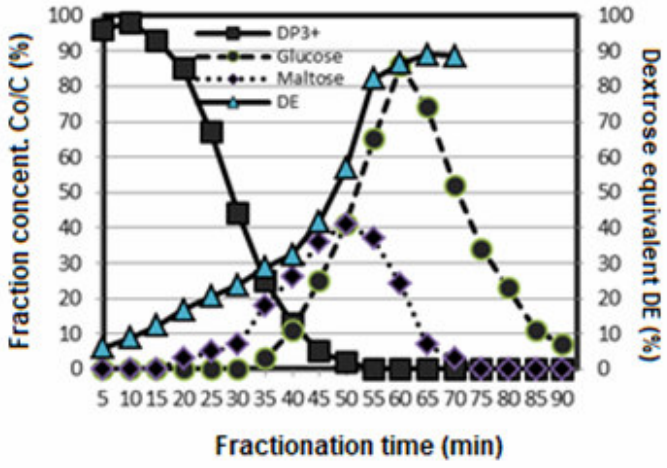

Figure 10. Separation profile of low molecular weight fraction from pyrodextrin hydrolysate of 50°Bx, space verocity of 0.5 BV by Lanlang® CS-1 resin column

4. CONCLUSION

Based on the results of this study, it can be concluded that it is possible to use ethanol precipitation and ion-exchange chomatography technology as well for enriching and recovering the indigestible fraction in pyrodextrin hydrolyzate. For the ethanol precipitation technology, the parameters include: Pyrodextrin solution concentration of 40%, rate of ethanol volume versus dry mass of pyrodextrin of 10 times (v/w), and time for separation precipitation of 5 hours. Strong acid cation exchange resins in Ca++ form are required for the ion-exchange chromatography technology, along with separation conditions such as space velocity SV 0.25 - 0.50 and an initial pyrodextrin solution concentration of 30 - 40°Bx. The product recovery by ethanol precipitation provides a high separation yield, while the ion-exchange column yields less at higher purity. It is necessary to improve and upgrade the technology and consider the econo-technical efficiencies to successfully choose the appropriate separation technology for production applications.

ACKNOWLEDGMENTS

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APPLYING RANDOM FORESTS ALGORITHM FOR LAND COVER MAPPING BASED SATELLITE IMAGERY

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ABSTRACT

Land cover mapping is vital for comprehending Earth's surface features, encompassing both natural (vegetation, hydrological systems) and man-made elements. It provides essential information for sustainable and responsible land management practices, helping to balance economic development with environmental conservation. There are several approaches to mapping land cover, such as: field surveys, geospatial analysis, or remote sensing. Each of these methods has its own strengths and limitation depending on factors such as the scale of mapping, availability of data, accuracy requirements, and budgetary constraints. In order to achieve the most accurate and detailed results, land cover mapping often involves a combination of these methods. This study proposed one of common machine learning algorithms - random forests to be used to classify landcover types automatically. This algorithm learned patterns and features from training data collected from a Landsat 8 scene of Phu Ly city, Ha Nam province, then applied them to classify unlabelled data of the whole scene. The approach yielded a remarkable 95% accuracy, surpassing alternatives such as a popular maximum likelihood supervised classification. Accurate land cover mapping facilitates decision-making, assesses land use changes, and supports sustainable land management. It provides valuable insights for environmental monitoring, urban planning, and biodiversity conservation. Thus, the random forests approach has shown promising results in land cover mapping, enhancing our understanding of Earth's dynamic landscape.

Keywords: *Land cover mapping, landsat 8, machine learning, maximum kikelihood, random forests.*

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1. INTRODUCTION

Land is one of the most important natural resources; is a home for all living species and human; and is place to host all physical, climatic and economic activities [1]. Land cover is referring to the surface covering over on the ground such as shrubland, built-up, water or vegetation, etc. The other term sharing similarities to land cover is land use, which refers to purposes of the assigned land, like agriculture, forestation, range land. Land use and land cover (LULC) commonly cohere to illustrate both humane activities and natural elements on the landscape. Industrialization, economic growth and urbanization are processes that mainly result changes the landscape over a specific time frame. Thus, it is important to conduct a better understanding of how land has been utilized as well as an involvement of land management policies and land monitoring to ensure sustainable development [2]. Mapping LULC over a specific time frame carries out a responsibility of supporting materials for land management by facilitating resource allocation, environmental monitoring, land use planning, natural resource management, and disaster risk assessment planning.

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There are several methods available for landcover mapping. Remote sensing involves using satellite or aerial imagery along with image classification algorithms to identify and classify different land cover types [3], [4]. Field surveys involve direct observations and data collection on the ground to validate and improve land cover maps [5], [6]. Geospatial analysis combines various spatial datasets, such as satellite imagery and GIS data, to generate or update land cover maps [7]. LiDAR technology utilizes laser beams to measure surface features, such as topography and vegetation structure [8], [9]. Data fusion combines information from multiple sensors or sources to enhance the accuracy and detail of land cover maps [10], [11]. Machine learning and artificial intelligence techniques use algorithms to automatically classify land cover based on training data [12], [13]. Unsupervised and supervised classification methods group pixels or objects in an image based on their spectral characteristics. The choice of method depends on factors such as scale, data availability, accuracy requirements, and budget constraints. Often, a combination of these methods is employed to achieve accurate and comprehensive land cover mapping results [14], [15].

In this study, we investigated in an integration of satellite image and one of machine learning methods – the random forests. Remotely sensed data are common resources for LULC mapping programs because of their advantages of providing many prospects to obtain physical statuses of LUCL at a certain or various spatial and temporal resolution [16]. Landsat images are more appropriate for mapping LULC at moderate scale (level 1 or level 2 of land classification systems respectively) within up to 90% of accuracy expectation of the Maximum Likelihood method (ML)– a very widely used technique of the image classification process [16]. However, there are other prospective methods that allows to get a higher accuracy of the analysis at some specific cases, such as "Random Forests technique" (RF) which uses a hierarchy of decision tree to assign samples into each class [17]. Therefore, we also examined how effective the random forests and ML supervised classification were in our case study. However, it is important to underline these factors in order to extract land cover information from satellite images: (1) a level of land classification system; (2) a requirement of accuracy; (3) a type of a chosen image; and (4) an image classification method.

Our study area is the entire Phu Ly city of Ha Nam province where rapid urbanization has been occurring. The pace of urban expansion in the area has led to an increased demand for frequent updates on land status and comprehensive insights into land management for local authorities. As the city experiences significant growth and development, it becomes crucial to have up-to-date information on land cover and land use patterns. Accurate and timely land cover mapping is essential to understand the dynamics of urbanization, monitor changes in land use, and support effective land management decisions. By providing a comprehensive and supporting maps using a new approach algorithm - Random Forest to process up-to-date remotely sensed data, this research is potentially expected to improve understanding of the current land status, including the distribution of urban, agricultural, residential, and other land cover types, our study aims to assist local authorities in making informed decisions, planning for infrastructure development, and ensuring sustainable land management practices in Phu Ly.

2. METHODOLOGIES

2.1. The process overview

The methodologies employed in this study utilizes Landsat 8 imagery and the random forest classification algorithm for land cover mapping. The following steps outlined he approach (figure 1 highlighted the whole process in a flowchart):

Step 1- Data Acquisition: Landsat 8 imagery was acquired for the study area, Phu Ly city of Ha Nam province, to capture the necessary spectral information required for land cover mapping. In this study, we acquired one Landsat scene of Phu Ly with the ID number was LC08_L2SP_

127046_20210717_20210729 from website: https://earthexplorer.usgs.gov/.

Step 2- Preprocessing: The Landsat 8 imagery was pre-processed to correct for atmospheric effects, sensor artifacts, and radiometric calibration. This ensures the data is in a suitable form for analysis.

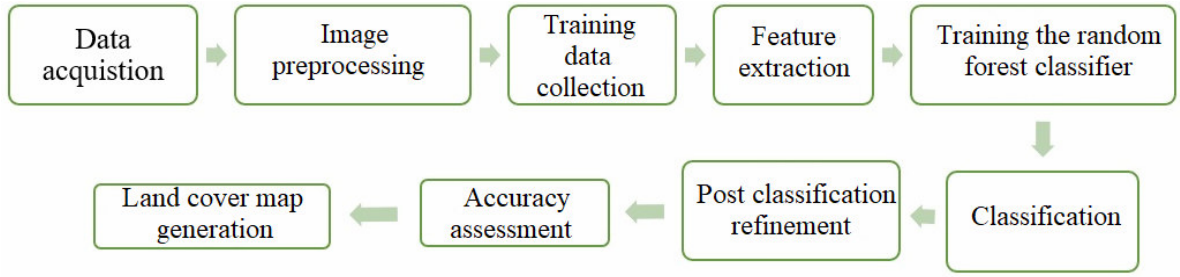


Figure 1. The flowchart described the 9 steps in mapping land cover using Landsat 8 scene and random forest of the study area.

Step 3- Training data collection: Ground-truth data or reference points representing different landcover classes were collected through field surveys or existing land cover datasets. These reference points served as training data for the random forest classifier.

Step 4- Feature extraction: Relevant spectral, textural, or spatial features were extracted from the pre-processed Landsat 8 imagery to characterize the different land cover classes. These features demonstrated the distinguishing characteristics of each land cover type.

From step 2 to step 4, we worked on Erdas software then summarized the data as checkpoints for the next steps of training and classifying in CART Navigator - Salford.

Step 5- Training the random forest classifier: The extracted features from the training data were used to train the random forest classifier. The algorithm learned the relationships between the spectral signatures and the corresponding land cover classes. Further information of this method was represented in the following section.

Step 6- Classification: The trained random forest classifier was applied to the entire Landsat 8 image, classifying each pixel into one of the predefined land cover classes based on its spectral characteristics. The classifier assigned a probability or confidence level to each class to quantify the uncertainty of the classification. In section 2.3, we discussed further the system applied in land cover classification.

Step 7- Post-classification refinement: Post-classification techniques, such as spatial filtering or object-based analysis, were employed to refine the land cover map, improve accuracy, and reduce classification errors.

Step 8- Accuracy assessment: The accuracy of the landcover map was assessed by comparing the classified results with independent reference data. This evaluation helped quantify the reliability and overall accuracy of the classification. We also conducted a set of training samples for the Maximum Likelihood to compare to our selected random forests.

Step 9- Landcover map generation: The final output was a land cover map that provides information about the distribution and spatial extent of different land cover classes within the study area, Phu Ly city.

2.2. Random Forests method

Random Forests (RF) is a machine learning algorithm to reduce number of training data relatively based on defined parameters [17]. The regression of training samples' numbers is practically depending on how to split the decision tree nodes. Decision trees generally are models constructed by a set of binary rules to estimate (predict and calculate) a target value [16]. There are two types of decision trees: regression tree (figure 2) and classification that are normally as known as CART (Classification and Regression Tree). However, in RF method, there will be "n"

CART that supports the whole process within less supervision of analysers [16].

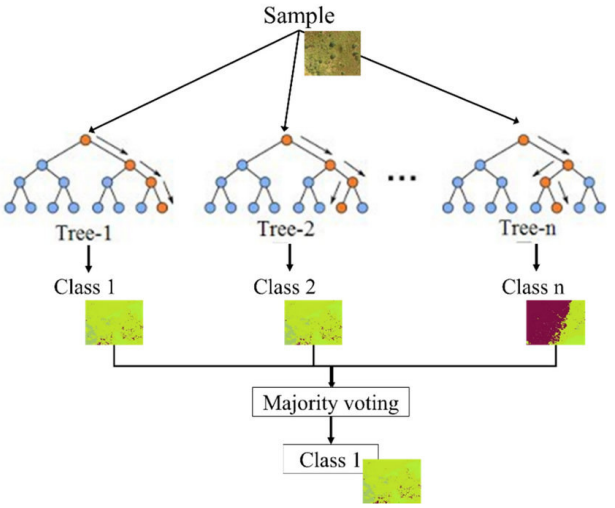


Figure 2. A general hierarchy of decision trees of the RF for classification [18]

To this experiment, we created totally 413 data points. Firstly, the surface reflectance of all bands for each checked point was extracted and converted to an ASCII file format. This file was then converted to an Excel file (.xls) format for compatibility with the Salford software. The Excel file contained approximately 413 points with indexes of band reflectance, class, and XY-coordinates. Figure 3 described how samples were assigned in decision trees and classified into class.

Next, the class variable was chosen as the target variable, and the band reflectance values served as predictors for testing the prediction success. A cross-validation process was conducted using the 413 values to assess the accuracy of the predictions.

The random forest algorithm was applied using the Salford software, which automatically generates decision trees with approximately 54 nodes. These decision trees had different relative costs and collectively contribute to the land cover mapping process.

By utilizing these steps, the land cover mapping process leveraged the band reflectance values as predictors and the class variable as the target, allowing for the generation of decision trees that aid in accurately classifying the land cover types within the study area. Figure 3 represented the CART at node 12 and 0.743 relative cost respectively.

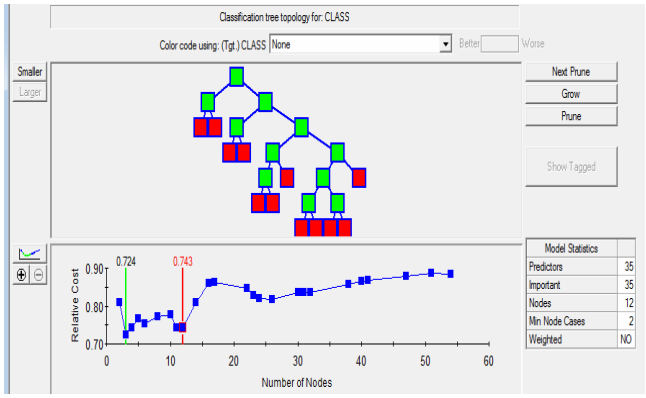


Figure 3. CART at node 12

Figure 4 shows a detailed example of how a classification tree applied in the experiment using red ban (band 3) and near infrared band (band 4) as parameters. The top of the tree was the "root node" evaluating the rule of "values in band 4 less than or equal to 40" would be designated into "water". Other decisions would need other rules until all branches end which means all pixels are assigned into land cover types.

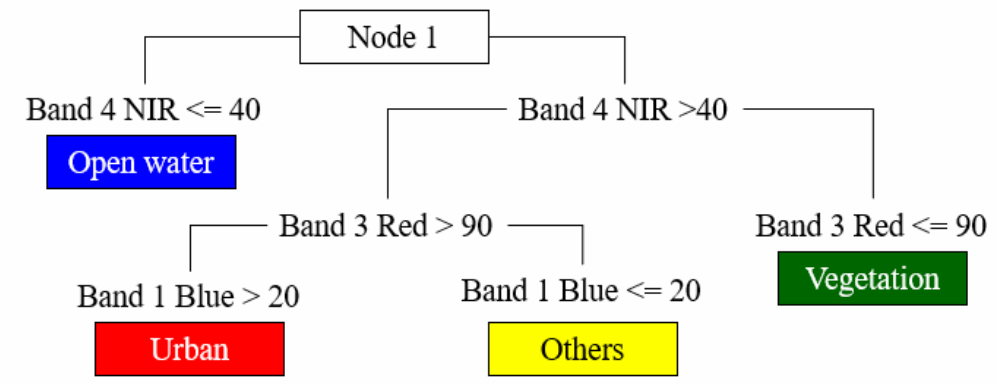
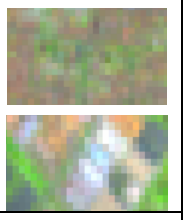
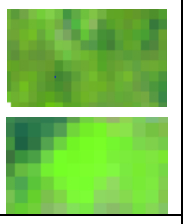
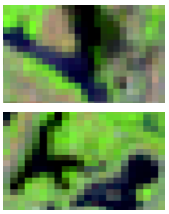
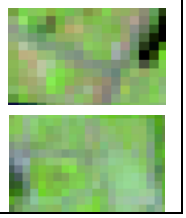


Figure 4. An example of Node 1 in a decision tree to assign the samples into a class

In this experiment, we used seven bands of a Landsat 8 image as seven nodes to construct decision trees due to the given conditions of moderate spatial resolution and level 1 of land classification system (4 land cover types). The nodes and trees would grow if there were more land cover types involved at more complicated classification system.

Additionally, we also conducted a Maximum Likelihood classification (ML) comparing how effective the RF method was. ML method is commonly used in image classification assigning pixels with a high potential similarity into class. The same training samples collection was carried out for both algorithms showed in table 1.

Table 1. Training samples for two classifications methods, RF and ML.

Landcover type	Indicators	Samples
Urban	*- Shape: Rectangles, blocks* *- Color: Brown, tan or gray* *- Distribution: Convergent or discreted*	
Vegetation	*- Shape: Non-homogenous* *- Color: Light green to dark green* *- Distribution: Convergent*	
Open water	*- Shape: linear or non-homogenous, somes are small size from 10-20 pixels* *- Color: Blue, dark blue to black* *- Distribution: Locating along side the boundary, interspred in urban areas*	
Others (a mix of roads and bareland)	*- Shape: Linear, non-homogenous* *- Color: Greenish gray* *- Distribution: Discreted or convergent around intersection*	

2.3. Land cover classification system

As followed by FAO [1], the land cover classification system (LCCS) involves two phases: "Dichotomous" and "Modular-Hierarchical" phase, where:

- The Dichotomous phase defines eight types of land cover: (1) Cultivated and Managed Terrestrial Areas; (2) Natural and Semi-Natural Terrestrial Vegetation; (3) Cultivated Aquatic or Regularly Flooded Areas; (4) Natural and Semi-Natural Aquatic or Regularly Flooded Vegetation; (5) Artificial Surfaces and Associated Areas; (6) Bare Areas; (7) Artificial Waterbodies, Snow and Ice; and (8) Natural Waterbodies, Snow and Ice.

- The Modular - Hierarchical phase designates landcover classes deriving from those 8 major pre-defined land cover types above, resulting a system of land cover types including:

- a *Boolean formula* showing each classifier used (all classifiers are coded);
- a *unique number* for use in Geographical Information Systems (GIS);
- a *name*, which can be the standard name as supplied or a user-defined name.

However, in this experiment, the chosen system was simplified from the FAO instruction based on the ideal of the map scale and the geographic area to determine the land types (legend) and mapping units (see figure 5). Thus, we chose the first level of land cover type classification, which are: Vegetation, urban, open water, and others.

- *Vegetation or vegetated area*: Where plants and trees mainly dominated;

- *Urban or residential areas*: Building and industrial infrastructure;

- *Open water*: Natural and artificial hydrological systems (lakes, ponds, rivers, streams);

- *Others or (sometime unclassified):* Roads, streets, bare soils.

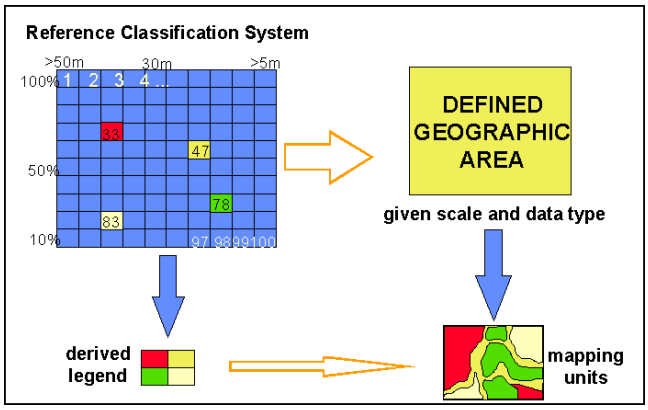


Figure 5. The FAO classification systems [1]

Figure 6. A Landsat composite image: vegetation (green), open water (dark blue); urban (brown)

3. RESULTS AND DISCUSSION

3.1. Study area

Phu Ly is a city located in Ha Nam province, Vietnam. It serves as the provincial capital and is situated approximately 60 kilometers (37 miles) to the southwest of Ha Noi, the country's capital. The city holds historical and cultural significance and features a blend of traditional Vietnamese elements and modern development.

Phu Ly is known for its scenic surroundings, with picturesque landscapes, rivers, and lush greenery. The city's urban area is characterized by a mix of old and new architecture, reflecting its evolving identity as it adapts to contemporary times while preserving its historical roots.

As the capital of Ha Nam province, Phu Ly serves as an administrative, economic, and cultural hub for the region. It's home to various government offices, educational institutions, markets, and commercial centers, contributing to its importance in the province's socio-economic activities.

Overall, Phu Ly is a city that encapsulates the harmonious coexistence of tradition and progress, offering a glimpse into Vietnam's past and present while playing a vital role in the development of its surrounding province.

3.2. Training data separability assessment

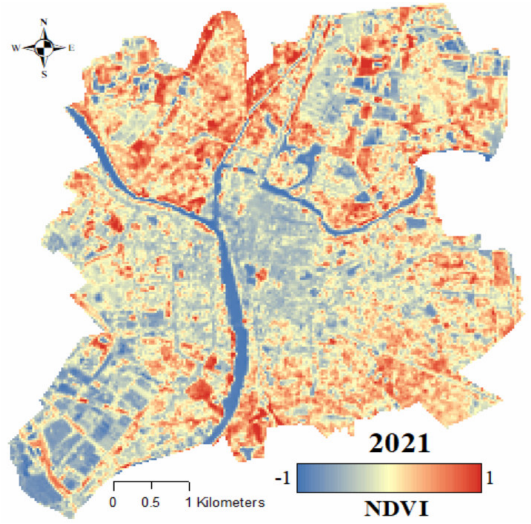


Figure 7. A NDVI image generated after processing, values are from -1 to 1

Data using to conduct experimental results are a Landsat 8 OLI image captured Phu Ly city of Ha Nam province in July 2021, local maps and other records for sampling process and accuracy assessment.

The Landsat 8 image was pre-processed to correct atmospheric and geometric effects, and to convert radiance value of each pixel to digital number (DN from 0-255) for the next step of the processing. Figure 6 illustrated the "false-color" composite image (5-4-1) in which different land cover types appears in various color scheme: bright green for vegetation, dark blue for open water and brownish tan for urban. Figure 7 was an extracted NDVI (Normalized Difference Vegetation Index) image after the pre-processing step, representing pixel values from -1 to 1. In LULC mapping, NDVI index is mainly used as one of indicators for selecting samples.

To assess the similarity and distinguishability of selected samples' spectra, we employed correlation charts depicting the spectral distribution of image channels. The results indicated that the chosen samples, after the verification process, exhibited good distinguishability, particularly in the "surface water or aquatic system" and "vegetation" classes. The "other land and urban" class was more challenging to differentiate due to certain pixels sharing spectral value similarities. This also clearly reflected the accuracy of classification results for each class and overall, for the maps. Figure 8 illustrated the spectral distribution space of pixels for the predicted samples in the red channel (3), near-infrared channel (4), and shortwave infrared channel (5). Pixels within urban areas (yellow) displayed a more diverse spectral dispersion compared to pixels belonging to other classes. Water and vegetation exhibit higher spectral concentration (with low spectral values across channels), while "other land" (red) has pixel values overlapping with the spectral distribution of the urban class.

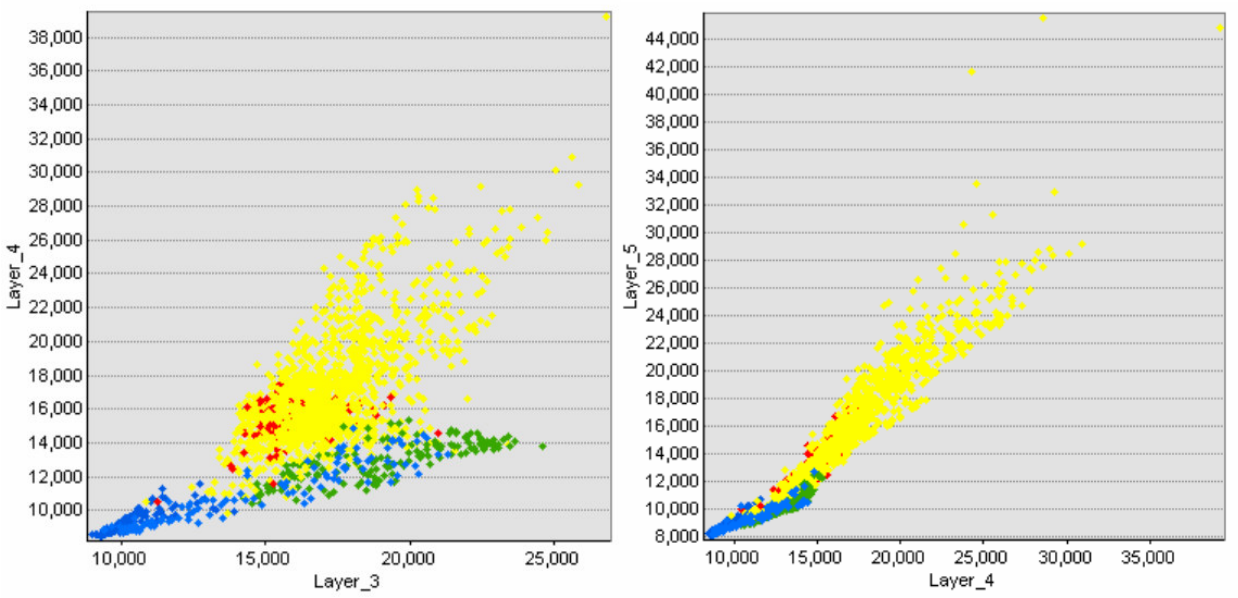


Figure 8. The spectral distribution graphs of different land cover classes (left: Red channel - layer 3 and near-infrared channel - layer 4; right: near-infrared channel - layer 5 and shortwave infrared channel - layer 5)

3.3. Land cover classification based on FAO system

The research investigated the efficiency of the Random Forest machine learning technique for land cover classification using Landsat 8 surface reflectance data at a 30-meter resolution. Figure 9 visually showcased the implementation of this algorithm for generating a land cover map in the year 2021. The outcomes of this analysis were obtained through the Erdas platform.

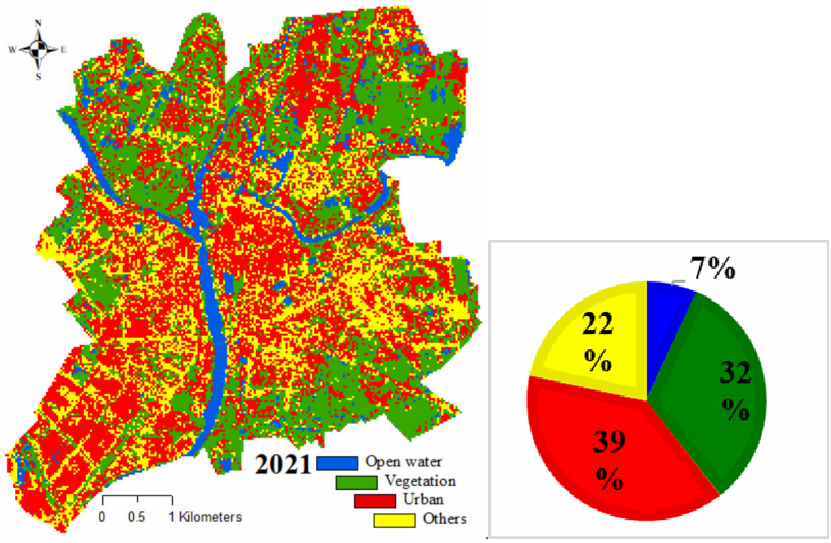


Figure 9. Map of land cover of study area in 2021 and an area chart

Due to the pie chart of figure 9, comparatively, the urban category comprised the largest proportion at 39%, followed by vegetation at 32%. Open water accounted for 7% of the area, and the remaining 22% was attributed to other land types.

3.4. Comparisons of different classification performances

Our results showed that in both methods, there was no un-classified land type. All pixels were assigned into desiring classes. Maps of landcover are illustrated in figure 10 to the RF method and figure 11 to the ML one, respectively. Generally, urban areas were covering more than 40% of the total while open water was less than 10%. In the RF method, roads and streets were captured more accurate of which occurence is more obviously linear (class of "other"). In the ML method, on the other hand, those road features scattered around urban areas without homogenous shapes resulting the indicator "shape" did not work well. It is understandable because the ML statistical algorithm is mainly based on spectal information of pixel. Therfore, separativity is not an easy step depending on the quality and condition of capturing images. This experienced image was collected in July 2021 which was in the rainy season, so soil was more moisturous leading to some lands could be assigned into "open water", for example of the lands on the south west of reseacher area on ML classified map while practically they were urbans.

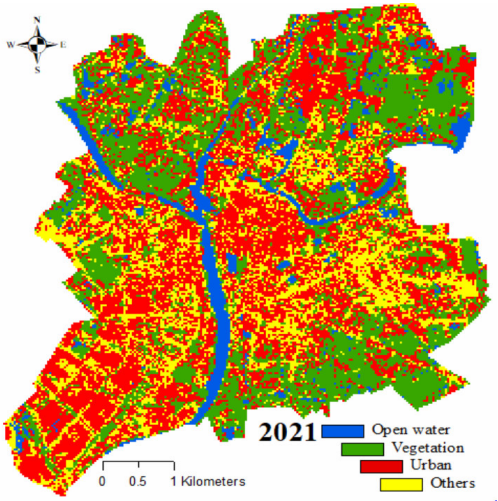


Figure 10. Random forest classification land cover map

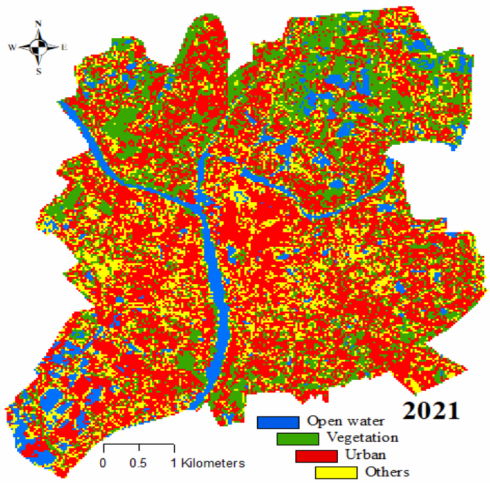


Figure 11. Maximum likelihood classification land cover map

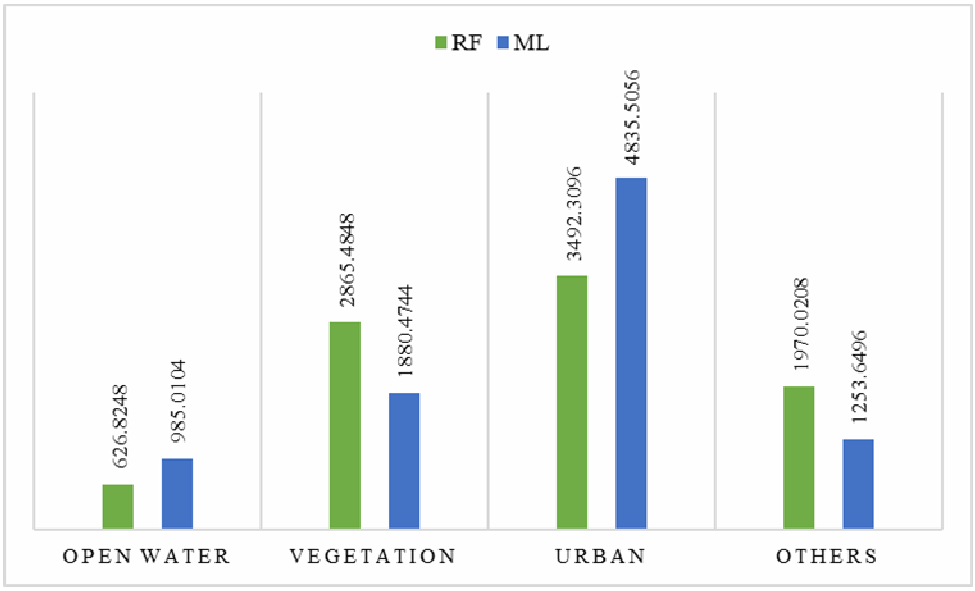


Figure 12. Area comparison of each class when performing random forest and maximum likelihood (unit:Hectares)

For the "Open water" category, the value was 626.82 ha under the RF technique, while it was 985 ha under the ML technique. This indicated that the ML technique yielded a higher value for this category.

In the "Vegetation" category, the value was 2865.48 ha with the RF technique, whereas it was 1880.47 ha with the ML technique. Here, the RF technique produced a higher value for this category.

Regarding the "Urban" category, the value was 3492.3 ha using the RF technique and 4835.5 ha using the ML technique. This suggested that the ML technique resulted in a higher value for this category.

Lastly, for the "Other land type" category, the value was 1970 ha with the RF technique, while it is 1253.65 ha with the ML technique. This implied that the RF technique produced a higher value for this category.

In summary, the ML technique generally yielded higher values for the "Open Water," "Urban," and "Other land types" categories, while the RF technique generated a higher value for the "Vegetation" category.

3.5. Accuracy assessment

The accuracy analysis was conducted to both processes with in 413 selected ground points using supporting materials. The overall accuracies of RF and ML method and are about 90% and 80% respectively providing that in this circumstance (Table 2), RF method was more effective than the ML. According to land cover types classification, water was shown to obtain higher accuracy comparing to the other types. Table 3 highlighted a 100% correction in water extraction from data point. Obviously, the RF method acquires more varilables (indicators) in the process, and there are more restrict rules to train and assign samples into classes. However, there needs a further evaluation of this RF analysis at higher level classification.

Table 2. Kappa coefficient and overall accuracy of each machine learning classifiers

Technique / Class	Random Forest (%)	Maximum Likelihood (%)
Open water	100	100
Vegetation	90	87
Urban	90	82
Other land types	85	77
Overall	90	83
Kappa	90	80

Table 3. The percentage of learning in evaluating the prediction success of each class, where water (class 1) has the highest percentage of 100 of accuracy

Actual Class	Total Class	Percent Correct (%)	1 N=12	2 N=77	4 N=245	8 N=78
1	5.00	100	5.00	0.00	0.00	0.00
2	66.00	53.03	1.00	35.00	23.00	7.00
4	50.00	73.54	6.00	40.00	214.00	31.00
8	412.00	80	0.00	2.00	8.00	40.00
Total	412					
Average		76.64				
Overall % Correct		71.36				

4. CONCLUSION

In overall, this research represented a potential approach of a machine learning method in image classification process for mapping land cover. Comparing to the common Maximum Likelihood, the random forests allowed to extract and generate information at a higher accuracy. They demonstrated robustness to noise and outliers, effectively handle high-dimensional datasets without overfitting, and exhibit high classification accuracy by capturing complex relationships in the data. The ability to assess variable importance helped identify influential features, while their capability to handle imbalanced data ensured accurate predictions for all classes. Additionally, random forests employed out-of-bag estimation for internal validation without the need for an additional validation dataset. However, they did have limitations, including limited interpretability due to their complex nature, computational intensity for large datasets, potential overfitting with noisy data, and challenges in extrapolation to unseen scenarios. Our future work will pay more attention on how to construct the next generation of decision trees and rules that satisfied a more detailed LULC map.

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PRESERVATION OF CHICKEN BREAST MEAT BY ROSEMARY (*Rosmarinus officinalis* L.)

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Doan Ngoc Thach Ky[1], Pham Duy Sang[1]

ABSTRACT

The study aims to extend the shelf - life of chicken breast meat by different treatments, including: (i) rosemary extract 14%, 16%, and 18%; (ii) rosemary essential oil 3%, 5%, and 7%; (iii) edible coatings formula (sodium alginate combined with rosemary extract or rosemary essential oil). The study evaluated the effect of these treatments on the physicochemical parameters (pH, color, shear force, drip loss) and microbiological indicators (*Escherichia coli* and aerobic plate count) of chicken breast meat during the cooling storage. The results showed that the physicochemical parameters of chicken breast meat were not be effected by different treatments, these parameters were good maintained during cooling storage (4 ± 1^0C). However, the antibacterial activity of rosemary extract was less effective to the growth of microorganisms, otherwise the rosemary essential oil 5% exhibits impressive antibacterial properties, including the growth of *E. coli* and aerobic plate count during seven days of storage. Moreover, the chicken breast meat was treated with sodium alginate - rosemary essential oil 5% had the *E. coli* and the aerobic microbial plate count within Vietnamese national standards (TCVN 12429-3: 2021) during ten days of cooling storage.

Keywords: *Chicken meat, essential oil, meat quality, rosemary, sodium alginate.*

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1. INTRODUCTION

Poultry meat is regarded as one of the most popular food items in the world, and its consumption expanded substantially during the previous two decades in the several nations [1]. Chicken meat has high content of protein and moisture, with weak acidic pH (5.3 – 6.5), therefore the chicken meat is sensitive to lipid oxidation and microbial development, leading to its shelf - life only 4 - 5 days [2], [3]. In order to prolong the storage time, natural ingredients are used to extend the shelf-life and increase the sensory quality of chicken meat. Rosemary (*Rosmarinus officinalis* L.), a *Lamiaceae* family member, is widely cultivated as an ornamental and aromatic plant worldwide [4]. It is known for its antibacterial and antioxidant properties based on its chemical components. Additionally, rosemary essential oil has been demonstrated to inhibit various foodborne pathogens from growing in vitro [5], [6]. The edible coating film is described as a thin layer of material that may be digested by people. Its function reduces the loss of volatile flavor compounds from food and provides a barrier to microorganisms [7], as well as improves esthetic appearance by minimizing the development of physical damage and improving the surface luster [8], [9]. For these reasons, the study was carried out to investigate the ability to prolong the shelf - life of chicken breast meat by using rosemary based on its antioxidant and antibacterial properties.

2. MATERIALS AND METHODS

2.1. Materials

Chicken breast meat and fresh rosemary were used in the experiment, which were purchased at the markets in Ninh Kieu district, Can Tho city,

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Vietnam. Fresh Rosemary tree was purchased at the shop "Dalat farm", the chicken breast meat was collected in the CP Freshmart (the time for samples collection was in the early morning when the shop received the newest products from the company). The commercial rosemary essential oil used in the study was sourced from India and distributed by Heber Vietnam Co., LTD.

The bacterial cultural mediums used in the study: Nutrient Agar (NA) - India, Tryptone Bile Glucuronic (TBX) Agar - India, Sodium alginate (SRK Entersprise, India).

2.1.1. Rosemary extract (RE) and rosemary essential oil (REO) preparation

Rosemary extract preparation: the branches of Fresh Rosemary tree were cut and cleaned with water and drained for 2 minutes. Rosemary was ground with cleaned water at the content such as: 14%, 16%, and 18% (w/w), then the mixture was filtered and collected clear rosemary extract.

Rosemary essential oil preparation: Emulsifier Tween 80 (Polysorbate 80) (10% mass fraction of mixture) was dissolved in distilled water, heated to 50^0C, stirred continuously for 5 mins to obtain a homogeneous solution and cooled down. Thereafter, the essential oil with different content such as: 3%, 5%, and 7% (v/v), that was slowly added to the solution and stirred for 10 minutes.

2.1.2. Process of treating chicken breast with rosemary extract, rosemary essential oil.

The chicken breast meat was bought at the market and brought to the lab to be pretreated with cleaned water and drained. Chicken breast will be immersed in rosemary extract or rosemary essential oil (prepared) for 60 seconds for each sample. The samples were placed in PA packages, vacuumed and sealed. The sample kept at 4 ± 1^0C for seven days, tested for microorganism and physicochemical properties on days 0, 2, 4, and 7.

2.1.3. Coating solution preparation

Sodium alginate solution of 2% (w/v) fixed, proceed to weigh 20 g of sodium alginate completely dissolved in 1 L of water (mixed in a temperature bath of 70^0C and stirred), cooling down to ambient temperature. Rosemary extract and rosemary essential oil (with concentrations selected from experiments 1 and 2) were added to the solution, then the mixture was homogenized to obtain a homogeneous coating solution. There were 3 types of coating solutions, including: sodium alginate (SA), sodium alginate and rosmary extract (SA-RE), sodium alginate and rosmary essential oil (SA-REO).

2.1.4. Process of treating chicken breast with sodium alginate edible coating

The chicken breast meat was bought at the market and brought to the lab to be pretreated with water and drained. Then immerse each sample in a coating mixture solution for 60 seconds. The samples storaged in PA packages, vacuumed and sealed, and kept at 4 ± 1^0C for ten days tested for microorganism and physicochemical properties on days 0, 3, 6, and 10.

2.2. Methods

2.2.1. Color assessment

The colors were measured using a colorimeter Fru (WR10, China). Determine the values of L*, a*, b*. The color deviation (ΔE) of meat after n days is calculated according to the formula:

$$\Delta E = [(L^*_0 - L^*_n)^2 + (a^*_0 - a^*_n)^2 + (b^*_0 - b^*_n)^2]^{1/2}$$

2.2.2. pH assessment

Using the Sinotester pH meter (pH - 618, China) to determine the pH value at different intervals. Mince 5 g chicken breast meat finely to ensure that both the inside and the outside are sampled, placed in a beaker and pour 50 ml of distilled water. Then measure the pH and record the value.

2.2.3. Shear force assessment: According to Young's Modulus formula: $E = \frac{P}{\Delta L/L}$ (Pa)

2.2.4. Drip loss assessment

Determination of drip loss rate (%) by weighing samples before and after the period of storage. Weigh meat samples and PA packaging before storage (m_1). After storage, pour out all the water, then reweigh the meat sample and PA

package (m_2). According to the formula: drip loss = $\frac{m_1 - m_2}{m_1} \times 100\ (\%)$

2.2.5. Aerobic plate count assessment: according to TCVN 4884-1: 2015 (ISO 4833-1: 2013) [10].

2.2.6. E. coli plate count assessment: according to TCVN 7924-2: 2008 (ISO 16649-2: 2001) [11].

3. RESUTLS AND DISCUSSION

3.1. Effect of rosemary extract on the physicochemical parameters of chicken breast meat during storage

During the preservation process, it is important and necessary to monitor the changes in the physicochemical parameters of chicken breast meat. Indicators such as color, pH, texture (shear force), and drip loss directly affect the quality. Tables 1 and 2 showed that all treated samples with the rosemary extract that had significantly lower L* values (lightness value) than the control sample ($p<0.05$), leading to these samples became darker. The decrease in L* value is due to the oxidation of meat, due to the myoglobin was oxidated to metmyoglobin (brown) during the storage [12]. When the content of rosemary extract treatment on the chicken breast was increased, as well as the prolonged storage time, the L* decreased, the b* value (yellowness) increased, ortherwise the a* values (redness) and ΔE value (color deviation) remained stable during 7 days of storage ($p<0.05$). Several research studies have indicated that rosemary extract has a possible antioxidant activity, leading to the stability of meat color [13 - 15].

Table 1. Effect of rosemary extract content on physicochemical properties of chicken breast meat

Content of rosemary extract (%)	L*	a*	b*	pH
Control	66.41 ± 0.50^{b}	3.40 ± 0.12^{c}	2.59 ± 0.26^{a}	6.19 ± 0.02^{a}
14	63.88 ± 0.50^{a}	2.86 ± 0.12^{ab}	11.86 ± 0.26^{b}	6.26 ± 0.02^{b}
16	64.85 ± 0.50^{a}	3.17 ± 0.12^{bc}	12.91 ± 0.26^{c}	6.23 ± 0.02^{ab}
18	63.55 ± 0.50^{a}	2.63 ± 0.12^{a}	14.98 ± 0.26^{d}	6.26 ± 0.02^{b}

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Table 2. Effect of rosemary extracts on physicochemical properties of chicken breast meat during storage periods

Days	ΔE	pH	Shear force (kPa)	Drip loss (%)
0	0.00 ± 0.53^{a}	6.23 ± 0.02^{ab}	15.28 ± 0.35^{c}	0 ± 0.56^{a}
2	2.64 ± 0.53^{b}	6.25 ± 0.02^{bc}	13.57 ± 0.35^{b}	5.43 ± 0.56^{b}
4	2.82 ± 0.53^{b}	6.29 ± 0.02^{c}	12.67 ± 0.35^{b}	5.84 ± 0.56^{b}
7	3.69 ± 0.53^{b}	6.18 ± 0.02^{a}	11.43 ± 0.35^{a}	6.44 ± 0.56^{b}

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Normally, the pH of fresh chicken meat ranges from 5.3 to 6.5 after slaughter [3]. Tables 1 and 2 showed that the pH value of treated chicken breast meat was higher than control sample, the difference was statistically significant ($p < 0.05$). But pH values of the treated samples still remained within the standards of TCVN 12429-1: 2018 [16].

The pH value was stable during storage but it decreased slightly on seventh day. Hosseini *et al.*

(2021) [17] reported that the pH decrease might be related to the dominance of lactic acid bacteria over time, which leads to the accumulation of acidic products in the extracellular environment, the primary cause of pH decline in meats.

The results of shear force and drip loss showed that the treatment of rosemary extract effected on the texture of chicken breast meat, and there was statistically significant difference ($p<0.05$) between the treated samples and the control. The shear force values decreased slightly, as well as drip loss increased during the storage period ($p<0.05$), water inside the meat is released, causing chicken breast meat to be softer (Table 2).

3.2. Effect of rosemary extract on the antimicrobial activity in chicken breast meat during storage

After slaughtering, chicken meat is a suitable environment for the growth of microorganisms, table 3 and table 4 show the *E. coli* and the aerobic plate count in the chicken breast meat that was treated at different rosemary extract content during storage period.

Table 3. The effect of content of rosemary extract on the *Escherichia coli* plate count in chicken breast meat

Content of rosemary extract (%)	*Escherichia coli* (10^3 cfu/g) by storage period (days)				
	0	2	4	7	Mean
Control	341.0	342.5	383.5	384.5	362.9^{c}
14	268.4	181.5	283.5	250.0	257.6^{b}
16	225.0	199.0	153.5	127.0	176.1^{a}
18	280.0	175.0	220.0	314.0	264.8^{b}
Mean	278.6^{b}	224.5^{a}	260.1^{b}	268.8^{b}	

Different letters in the same column or row represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

In the table 3 and 4, the results showed the antibacterial activity of rosemary extract, the statistically significant difference among control sample and treated samples was found on the *Escherichia coli* plate count (Table 3), and the aerobic plate count (Table 4). The sample was treated by 16% of rosemary extract that had the best antibacterial activity, the *E. coli* count and aerobic plate count were the lowest. Sienkiewicz *et al.* (2013) [18] revealed rosemary has potential effectiveness against extended-spectrum β-lactamase positive bacteria as well as resistant clinical strains of *Escherichia coli*. Because of the combined effects of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosic acid, rosmanol, and isorosmanol, rosemary has an inhibiting impact on microbial. They interact with the cell membrane, changing the creation of fatty acids, the transport of electrons, the leakage of cellular components, the genetic material and nutrients [19]. Also, Govaris *et al.* (2010) [20] discovered rosemary extract inhibited the increase of total viable counts in poultry breast meat during storage, the antibacterial activity of phenolic compounds was associated with the inactivation of cellular enzymes [21], [22]. However, the results in table 3 and 4 showed that, both of the *Escherichia coli* and the aerobic plate count were higher than the standard of TCVN 12429-3: 2021 [23] throughout 7 days of cooling storage, indicating the antibacterial activity of rosemary extract was less effective to the growth of microorganisms. It might due to the content of rosemary extract was not high enough and contained a little amount of the antibacterial components.

Table 4. The effect of content of rosemary extract on the aerobic plate count in chicken breast meat during storage period

Content of rosemary extract (%)	Aerobic plate count (10^6 cfu/g) by storage period (days)				
	0	2	4	7	Mean
Control	23.0	49.0	48.5	57.0	44.8^{c}
14	25.5	29.0	28.5	19.0	25.3^{b}
16	14.5	18.5	17.5	15.0	17.4^{a}
18	15.0	17.5	16.0	23.0	26.8^{b}
Mean	19.5^{a}	28.5^{b}	27.6^{b}	28.5^{b}	

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

3.3. Effect of rosemary essential oil (REO) on the physicochemical parameters of chicken breast meat during storage

The results on the tables 5 and 6 showed that the treated samples with rosemary essential oil had higher L* values than the control sample and tended to increase the brightness throughout storage ($p<0.05$). The sample was treated with 5% REO that had a lower a* value than the other samples. The redness value tends to increase compared to the beginning of storage period, Kahraman *et al.* (2015) [24] also reported that rosemary essential oil treatment significantly increased the a* value of stored poultry fillets. Most of the b* values of the treated samples with REO were higher than the control sample ($p<0.05$). The color deviation remained stable until day 7 ($p<0.05$), because the essential oil had antioxidant properties, therefore the color of chicken breast meat was stable. Moreover, the pH value of chicken breast meat was also affected by REO treatment, there was statistically significant difference pH value of chicken breast meat during storage time.

Table 5. Effect of rosemary essential oil content on physicochemical properties of chicken breast meat

Content of rosemary essential oil (%)	L*	a*	b*	pH
Control	66.41 ± 0.50^{b}	3.40 ± 0.12^{c}	2.59 ± 0.26^{a}	6.19 ± 0.02^{a}
3	63.88 ± 0.50^{a}	2.86 ± 0.12^{ab}	11.86 ± 0.26^{b}	6.26 ± 0.02^{b}
5	64.85 ± 0.50^{a}	3.17 ± 0.12^{bc}	12.91 ± 0.26^{c}	6.23 ± 0.02^{ab}
7	63.55 ± 0.50^{a}	2.63 ± 0.12^{a}	14.98 ± 0.26^{d}	6.26 ± 0.02^{b}

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Table 6. Effect of rosemary essential oil on physicochemical properties of chicken breast meat during storage period

Days	Δ E	pH	Shear force (kPa)	Drip loss (%)
0	0.00 ± 0.53^{a}	6.23 ± 0.02^{ab}	15.28 ± 0.35^{c}	0 ± 0.56^{a}
2	2.64 ± 0.53^{b}	6.25 ± 0.02^{bc}	13.57 ± 0.35^{b}	5.43 ± 0.56^{b}
4	2.82 ± 0.53^{b}	6.29 ± 0.02^{c}	12.67 ± 0.35^{b}	5.84 ± 0.56^{b}
7	3.69 ± 0.53^{b}	6.18 ± 0.02^{a}	11.43 ± 0.35^{a}	6.44 ± 0.56^{b}

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

The table 6 showed the texture of chicken breast meat changed during the storage time, the shear force value decreased when the storage time was extended. Water is tightly bound to proteins in muscle tissue and is also present in the spaces between myofibrils, giving the structure of meat was soft and elastic [25], part of water was lost during storage, causing the meat to degrade its elasticity and become more tender. The drip loss increased until day 7, due to the high content of essential oil was added, lead to the protein structure was broken down and the leakage water increased.

3.4. Effect of rosemary essential oil on the antimicrobial activity in chicken breast meat during storage

The results in table 7 showed that the treated samples with higher content of rosemary essential oil had lower *E. coli* plate count than others ($P<0.05$). Ojeda-Sana *et al.* (2013) [26] proved that rosemary oils include α-pinene, which has a broad antibacterial spectrum against Gram-negative and Gram-positive bacteria, as well as 1,8-cineole, which has antibacterial action against Gram-negative pathogenic bacteria by disrupting cell membranes. Many studies have aimed to clarify the antimicrobial mechanism of essential, including cell wall destruction, cytoplasmic membrane disturbance, disruption of the proton motive force, coagulation of cell contents, ATP hydrolysis, and a decrease in the synthesis process, resulting in a drop in the intracellular pool of ATP [27], [28]. The results of table 7 showed that there was significant statistical difference between storage days, the *E. coli* plate count continuously increased during 7 days of storage.

Table 7. The effect of content of rosemary essential oil on the *Escherichia coli* plate count in chicken breast meat

Content of rosemary essential oil (%)	*Escherichia coli* (10^3 cfu/g) by storage time (days)				
	0	2	4	7	Mean
Control	126.6	191.8	197.2	236.8	184.85^{d}
3	47.2	111.6	113.4	143.4	107.15^{c}
5	16.1	5.0	3.0	0.6	6.18^{a}
7	12	12.2	14.7	17.1	14.28^{b}
Mean	50.83^{a}	80.65^{b}	82.44^{b}	99.54^{c}	

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Increasing the content of rosemary essential oil treatment, the aerobic microbial count tended to decrease (Table 8). This is analogous to the study of Hać-Szymańczuk *et al.* (2017) [29], who reported that the total bacterial count was significantly lower in samples with added rosemary essential oil and ethanol extract (70% v/v) compared to the control sample. The table 8 showed a large microbial count of the control sample had proliferated grown on day 7 but the samples treated with REO at content of 5%, and 7% still inhibited the growth of aerobic microbial. All of samples were treated by REO, which had aerobic plate count within the standard of TCVN 12429-3: 2021 throughout storage. The results of table 8 showed that there was significant statistical difference between storage days, the aerobic plate count continuously increased during 7 days of storage. In particularly, the sample was treated by REO 5% that inhibited the growth of microorganisms, which had the *E. coli* and the aerobic plate count within the standard of TCVN 12429-3: 2021 [23] throughout seven days of cooling storage.

Table 8. The effect of content of rosemary essential oil on the aerobic plate count in chicken breast meat during storage period

Content of rosemary essential oil (%)	Aerobic plate count (10^5 cfu/g) by storage time (days)				
	0	2	4	7	Mean
Control	154.5	194.0	195.0	950.0	373.3^{c}
3	11.0	47.5	55.0	19.0	33.1^{b}
5	23.5	8.5	6.5	0.4	9.6^{a}
7	22.5	9.5	6.0	5.0	10.7^{a}
Mean	52.7^{a}	64.8^{b}	65.8^{b}	243.5^{c}	

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

3.5. Effect of sodium alginate edible coating incorporated with rosemary extract and rosemary essential oil on the physicochemical parameters

The results are indicated on tables 9 and 10, the L* values of edible coating formulas increased from 0 to day 6 and remained stable until day 10 ($p<0.05$). Both of the samples coated with aginate and rosmary (SA-RE and SA-REO) had lower a* values than samples coated with only alginate (SA). The results proved that the alginate coating contributes as an antioxidant barrier on preserving chicken breast meat. Samples treated with sodium alginate-rosemary extract (SA-RE) was the highest b* values, Lefebvre *et al.* (2021) [30] revealed that the rosemary plant contains pigments (i.e. carotenoids, chlorophylls) that may cause a color problem during the use of the extract. Extending the storage time, the color deviation tends to increase, statistically different from beginning. Besides, the pH tends to rise with a statistically significant difference ($p<0.05$), related to the ionized carbonate releasing from the coating [31].

Table 9. Effect of edible coating incorporated with rosemary on physicochemical properties of chicken breast meat during 10 days of cooling storage

Treatment	L*	a*	b*	pH	Shear force (kPa)
SA	63.24 ± 0.29^{b}	3.18 ± 0.11^{c}	3.43 ± 0.18^{a}	6.39 ± 0.02^{c}	15.02 ± 0.27^{b}
SA - RE	61.31 ± 0.29^{a}	2.33 ± 0.11^{a}	8.45 ± 0.18^{c}	6.26 ± 0.02^{b}	13.68 ± 0.27^{a}
SA - REO	63.44 ± 0.29^{b}	2.68 ± 0.11^{b}	3.84 ± 0.18^{b}	6.11 ± 0.02^{a}	14.04 ± 0.27^{a}

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Table 10. Effect of edible coating incorporated with rosemary on physicochemical properties of chicken breast meat during cooling storage period

Days	Δ E	pH	Shear force (kPa)	Drip loss (%)
0	0.00 ± 0.45^{a}	6.11 ± 0.03^{a}	16.23 ± 0.32^{d}	0.00 ± 0.47^{a}
3	2.84 ± 0.45^{b}	6.34 ± 0.03^{c}	15.19 ± 0.32^{c}	4.71 ± 0.47^{b}
6	4.57 ± 0.45^{c}	6.38 ± 0.03^{c}	13.43 ± 0.32^{b}	7.15 ± 0.47^{c}
10	3.97 ± 0.45^{bc}	6.20 ± 0.03^{b}	12.13 ± 0.32^{a}	7.14 ± 0.47^{c}

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Tables 9 and 10 show the differences between samples treated with different membrane formulations. Meat samples had a firm texture, and samples coated with only alginate (SA) that had greater shear force value than samples coated with alginate and rosmary (SA-RE and SA-REO). It is probably due to the effect of rosemary extract and rosemary essential oil to make the meat texture softer. Based on the ability of the alginate coating can reduce water loss from the cells, the drip loss values are relatively stable.

3.6. Effect of sodium alginate edible coating incorporated with rosemary extract and rosemary essential oil on the antimicrobial activity in chicken breast meat during cooling storage

The results from table 11 showed that samples were treated by SA-REO and SA coatings showed a very positive effect, the *E. coli* plate count was low, and the microbial resistance of these samples was better than the sample treated with SA-RE coating. Antimicrobial films improved the microbiological quality characteristics of coated chicken breast fillets during storage, extending their shelf life in cooling conditions [32]. The number of *E. coli* in the table 11 showed a downward trend compared to the first day ($p<0.05$).

The coating formulation combining sodium alginate with rosemary essential oil showed outstanding antimicrobial resistance in this experiment. The functionality of edible coatings can be enhanced by incorporating antimicrobial agents to protect food products from microbial pathogens and deteriorations, extend shelf life, and improve food safety [33], [34]. Coated treatment method showed optimal effectiveness, improving the disadvantage of uncoated treatment in preventing the growth of total aerobic microorganisms. Table 12 showed that samples treated with SA-REO had the lowest aerobic plate count ($p<0.05$) during ten days of storage. Its aerobic bacterial plate count remained relatively constant throughout the storage time and still belongs to the standard of TCVN 12429-3: 2021 [23].

Table 11. Effect of edible coating formulas on the *Escherichia coli* plate count

Treatment	*Escherichia coli* (10^3 cfu/g) by storage time (days)				
	0	3	6	10	Mean
SA	141.2	11.4	34.1	12.8	49.8^{b}
SA - RE	218.4	102.0	71.0	34.3	106.4^{c}
SA - REO	38.4	11.8	9.6	2.7	15.5^{a}
Mean	132.6^{a}	41.3^{b}	38.1^{b}	16.6^{c}	

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

Table 12. Effect of edible coating formulas on the aerobic plate count

Treatment	Aerobic plate count (10^5 cfu/g) by storage time (days)				
	0	3	6	10	Mean
SA	11.7	15.0	29.4	81.0	34.26^{b}
SA - RE	43.6	57.7	62.0	64.4	56.41^{c}
SA - REO	4.8	2.8	2.6	2.5	3.26^{a}
Mean	20.13^{a}	25.37^{b}	31.45^{c}	49.3^{d}	

Different letters in the same column represent significant differences between the investigated treatments at the 95% confidence level according to the LSD test

4. CONCLUSION

Rosemary extract, rosemary essential oil, and the combination between rosmary and edible coatings effectively stabilize physicochemical parameters of chicken breast meat, such as color, pH, texture, and drip loss throughout the cooling storage. In the antimicrobial aspect, the chicken breast meat was treated by 16% rosemary extract, or 5% rosemary essential oil, or alginate-rosemary essential oil that effectively reduced the density of microorganism than other treatments. In particular, chicken breast meat was treated with 5% rosemary essential oil had *E. coli* and the aerobic plate count within the standard of TCVN 12429-3: 2021 until seven days of cooling storage, while the chicken breast meat was treated with sodium alginate-rosemary essential oil had the *E. coli* and the aerobic plate count within Vietnamese national standards of TCVN 12429-3: 2021 until ten days of cooling storage.

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MICROSATELLITES POLYMORPHISM ASSOCIATED WITH HEPCIDIN/HAMP GENES POTENTIAL FOR SELECTIVE BREEDING OF DISEASE-RESISTANT BY *Streptococcus agalactiae* IN NILE TILAPIA IN VIETNAM

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ABSTRACT

Streptococcosis is considered an infectious disease causing significant economic loss in tilapia aquaculture in the world. Fish hepcidin is known as a hepatic antimicrobial peptide (HAMP), and is associated with innate immunity, which defends against various bacterial infections and viruses. Sixty Phu Ninh tilapia after the challenge test with *Streptococcus agalactia* at a dose of $2{,}2x10^6$ cfu/ml were sampled to analyse microsatellite/SSRs polymorphism in the hepcidin/HAMP genes and the resistance to *S. agalactia*. This study examined the relationship between seventeen microsatellite/SSRs polymorphism in the hepcidin/HAMP genes and the resistance to *S. agalactia* in the Phu Ninh tilapia strain. The results indicated that 13/17 hepcidin/HAMP-related SSRs were polymorphic markers based on the number of alleles, effective alleles, observed heterozygosity, expected heterozygosity, polymorphism information content, and Hardy-Weinberg equilibrium. In this study, there was a significant difference in genotype and allele frequency between two groups (alive fish and dead fish after challenging with *S. agalactiae*) for four SSRs (SSR3, SSR5, SSR14, and SSR16) ($p < 0.05$). These four SSRs are utilized as marker-assisted selection (MAS) in the breeding of tilapia for *S. agalactiae* resistance.

Keywords: *Disease-resistance, hepcidin/HAMP gene, Microsatellite, Phu Ninh tilapia strain, Streptococcus agalactiae*

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1. INTRODUCTION

Tilapia is among the world's most important aquaculture finfish species. Recently, streptococcosis is a bacterial disease caused by *Streptococcus* spp in tilapia culture. This disease has been recognized as one of the most serious bacterial diseases and caused high mortality. It is reported that the antimicrobial peptide hepcidin/HAMP genes were resistant to both gram-negative, gram-positive bacteria, and viruses. Chen *et al.* (2022) [1] identified 18 hepcidin genes in Nile tilapia, of which 12 hepcidin/HAMP genes were in the linkage group 11 (LG11), 6 hepcidin genes in two unplaced contig 825 and contig 1099. 12 hepcidin/HAMP genes in the LG11 encoded 4 HAMPs (eight HAMP1 genes, one HAMP2 gene, one HAMP3 gene, and two HAMP4 genes. Two hepcidin-like antimicrobial peptides (TH1-5 and TH2-3) were obtained in Mozambique tilapia. TH1-5 and TH2-3 were reported to defend against some bacteria. The translated regions of these two peptides contained 88, and 91 amino acids, respectively [2], [3]. The TH1-5 peptide was reported as an antimicrobial peptide against both

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gram-negative and gram-positive bacteria. Transgenic zebrafish with TH1-5 peptide resisted against *Vibrio vulnificus* (gram-negative bacteria) and *S. agalactiae* (gram-positive bacteria) [3]. Gong *et al.* (2016) [4] revealed that three hepatic antimicrobial peptides (HAMP1, HAMP2, HAMP3) were strongly expressed in the liverand the spleen of Nile tilapia in responding to *S. inae* infection.

Microsatellites, also named simple sequence repeats (SSRs), consist of short tandem repeat units of 1-6 base pairs (bp) in length [5]. SSRs are often highly polymorphic, abundant distribution throughout genome, codominant inheritance, and small size. Microsatellites have become a useful tool in population genetic analysis, genetic mapping, and marker-assisted selection. Microsatellites can be used to assess genetic diversity and develop molecular-breeding tools in fish. Microsatellites associated with disease resistance have been reported in several important aquatic species. In Japanese flounder (*Paralichthys olivaceus*), 50 SSRs were used to study lymphocytic disease resistance (LD-R). In which only the Poli9-8TUF locus was reported to be associated with LD-R [6]. In rock bream (*Oplegnathus fasciatus*), three potential microsatellite markers (CA3-05, CA3-06, and CA3-36) were suggested to be used to facilitate the selection of rock bream iridovirus-resistant (RBIV-resistant) [7].

This study aims to determine the relationship between microsatellite/SSRs polymorphisms and *S. agalactiae* disease-resistance in Phu Ninh tilapia strain.

2. MATERIALS AND METHODS

2.1. Materials

The 2nd selection generation on growth performance (Phu Ninh strain) of Nile tilapia (*Oreochromis niloticus*), from the Tilapia Research Centre (TILACEN), Research Institute for Aquaculture No.1 (RIA.1) was used in this study. This tilapia strain was offspring from two full-sib families and one half-sib family produced from November to December 2019. Tilapia (with size 25-30g/fish) was challenged with *Streptococcus agalactiae* 015-RIA1 (GenID: OK047709.1) in LD_{50} dose (2,2 x10^6 cfu/ml) after 21 days. The pectoral fin samples of Nile tilapia were collected from the dead and survival groups (30 samples/group) after the challenge. The fin samples were preserved in 96% ethanol and stored at 4°C for long-term storage.

2.2. DNA extraction

The genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's recommendations. The total DNA quantity and quality were measured using spectrophotometer NanoDropĂ 2000 (Thermo Scientific, USA) and analyzed by electrophoresis on a 0,8% agarose gel.

2.3. Microsatellite amplification and genotyping

Seventeen microsatellite-specific primers in hepcidin/HAMP genes reported by Chen *et al.* (2022) [1] were used in this study (shown in Table 1). These microsatellite-specific primers were designed by an online tool (Websat, http://wsmartins.net/websat/accessed on 7 May 2015). The primer length was 22 bp, Tm was 60°C, GC was 60%, and the product length was between 100 and 400 bp. All SSRs were located in intron or 5′- and 3′- flacking regions within/linked to HAMP genes in LG11 (as table 1).

The primer sets (Multiplex) of 17 SSRs were designed by Multiplex Manager software based on the annealing temperature of each primer pair [8]. The primer dimer detection was checked by an online tool (Multiple Primer Analyzer, Thermo Scientific, UK: https://www.thermofisher.com/vn/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html).

A total of four multiplex PCR sets were created, including three multiplex PCR with tetraplex SSRs (multiplex I, III, and III) and one multiplex PCR with pentaplex SSRs (multiplex IV) as shown in table 1.

Table 1. The information on seventeen microsatellite/SSRs used in this study

SSRs	Gen/ID	Primer sequences (5'-3')	Motif	Size (bp)	Dyes
Multiplex I:					
SSR4	HAMP2 3'end 4.3kb (ID: 100698871)	F: CACCACTGTCAACTGGCTAATG R:GTTACCTTCTTGATACCGCAGG	$(CTAC)_7$	359	FAM
SSR5	HAMP2 3'end 4.7kb (ID: 100698871)	F: CTTTGGGTAGAGGAACACTCCA R:TGCAGGTCAATAGCAATACCAC	$(TG)_{21}$	309	HEX
SSR9	HAMP1 5'end 1.3kb (ID: 109204256)	F: GCGCTGTATAAGATTCCCGTTA R:GGAAACACAAGAGACATGAGCA	$(T)_{20}$	370	TAM
SSR14	HAMP1 reverse strand 3'end 6.2kb (ID: 100534415)	F: TCGGAATTGAGCATTAAGACCT R:GGTTCCCCATAGAACTCCTTTT	$(TG)_{22}$	245	ROX
Multiplex II:					
SSR6	HAMP1 5'end 2kb (ID: 109204280)	F: CAGTGGGTGTTTGTTCCTTACA R:TAGTAGGCTTTGTGTGCATTCC	$(GT)_{41}$	315	HEX
SSR8	HAMP1 5'end 1.3kb (ID: 109204256)	F: CCCCATAGCACTCCTTTTATTG R:TCATTTGGAGGTGTTTTCACAG	$(TG)_{22}$	322	ROX
SSR10	HAMP1 3'end 7.6kb (ID: 109204256)	F: GTTTGTAGCTTAACCCATTCGC R:TGCCTTTGTTTAGATGAACTGC	$(TG)_{10}$	364	FAM
SSR17	HAMP1 3'end 4.2kb (ID: 109204285)	F: GGTTCCCCATAGAACTCCTTTT R:TCGGAATTGAGCATTAAGACCT	$(TG)_{15}$	261	TAM
Multiplex III:					
SSR1	HAMP2 5'end 3.8kb (ID: 100698871)	F: CCACACAATCAACACACTGGTA R:TCAAACAGAAACAGGGACACAC	$(TA)_{11}$	375	HEX
SSR2	HAMP2 5'end 3.8kb (ID: 100698871)	F: GCACAGACACAGTAACACATGC R: ACTCCCTGGTACATGCTTCCTA	$(CAGG)_6$	368	FAM
SSR13	HAMP1 reverse strand 5'end 6.3kb (ID: 109204255)	F: TTAAAATGGGCTCAGGAGAAAG R:ACACACATAGATTCATCCGCAC	$(ATTC)_7$	332	ROX
SSR16	HAMP1 reverse strand intron2 (ID: 100534415)	F: GTGGGAAACACAAGAGACATGA R:CAGGGCTGAGATAGATTTTGGT	$(T)_{23}$	232	TAM

Multiplex IV:					
SSR3	HAMP2 5'end 3.8kb (ID: 100698871)	F: TAGGAAGCATGTACCAGGGAGT R: AAAATCACTCAACCGTGTCCTT	$(GT)_{13}$	348	FAM
SSR7	HAMP1 3'end 4.7kb (ID: 109204280)	F: CCACACGCACTCTCACCA R:CTGGTAGCCTTGACCCAGTTT	$(AC)_{12}$	236	HEX
SSR11	HAMP4 5'end 1kb (ID: 109204092)	F: AAGTGTCGTTCCACCCACAT R:ACAGAGTGTTCTGGCTTTCACA	$(TG)_{29}$	382	TAM
SSR12	HAMP1 reverse strand 5'end 2kb (ID: 109204255)	F: GTGCATTACAGAGTGTTCTGGC R: GTCTGGAGCCAAGTGTCGTT	$(TG)_{27}$	395	HEX
SSR15	HAMP1 reverse strand 3'end 750bp (ID: 100534415)	F: CAACAAGTGAAGCGACCATATT R:TCACACACAAGCAGGTCAATTA	$AC)_{13}$	300	ROX

The PCR was carried out in a 25 µl total reaction using 2× My Taq™ HS Mix (Meridian Bioscience, Germany), 0.32 µM of each primer, 100 ng/µl of gDNA, and ddH_2O. PCR amplification was performed on the Mastercycler proS (Eppendorf, Germany) under the following conditions: Initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, and a final extension of 72°C for 2 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel with SafeViewTM Classic (ABM, Canada).

For the genotyping, the PCR products were analyzed fragments on the ABI (FirstBase, Malaysia). After the analysis, GeneMarker software V.2.2.0 was used to analyze allele sizes.

2.4. Determination of microsatellite/SSR polymorphisms

Evaluation of polymorphisms of SSRs is the first step to identifying potential SSRs associated with resistance to *S. agalactiae*. The polymorphism of the SSRs marker was evaluated on 60 individuals of the Phu Ninh strain. The evaluation was based on the following parameters:

- The polymorphism information content (PIC) was calculated following instruction on the Website (https://www.gene-calc.pl/pic). The PIC value of each SSR was determined automatically based on the number of alleles and their distribution frequency.

- Population genetic parameters (number of alleles-N at each microsatellite site, observed heterozygosity-Ho, expected heterozygosity-He and Hardy-Weinberg Equilibrium Assessment-HWE) were analyzed by using the software GenAlex 6.5-Genetic Analysis in Excel [9].

2.5. The identification of potential microsatellite/ SSRs associated with disease resistance

To determine potential SSRs associated with disease resistance by *S. agalactiae*, IBM SPSS Statistics version 20.0 software (SPSS Inc., Chicago, IL, USA) was used. The Chi-square tests ($p<0.05$) were performed on genotype and allele frequencies between two groups of live-dead after challenging test (30 samples/group of fish) for each polymorphic SSR

3. RESULTS AND DISCUSSION

3.1. PCR optimization

PCR production of each microsatellite/SSRs and Multiplex PCR is shown in Figures 1 and 2.

Figure 1. Gel electrophoresis of PCR product of seventeen SSRs

Lane 1 to15 are PCR products of SSRs; lane 16: Negative samples; Ladder 100bp

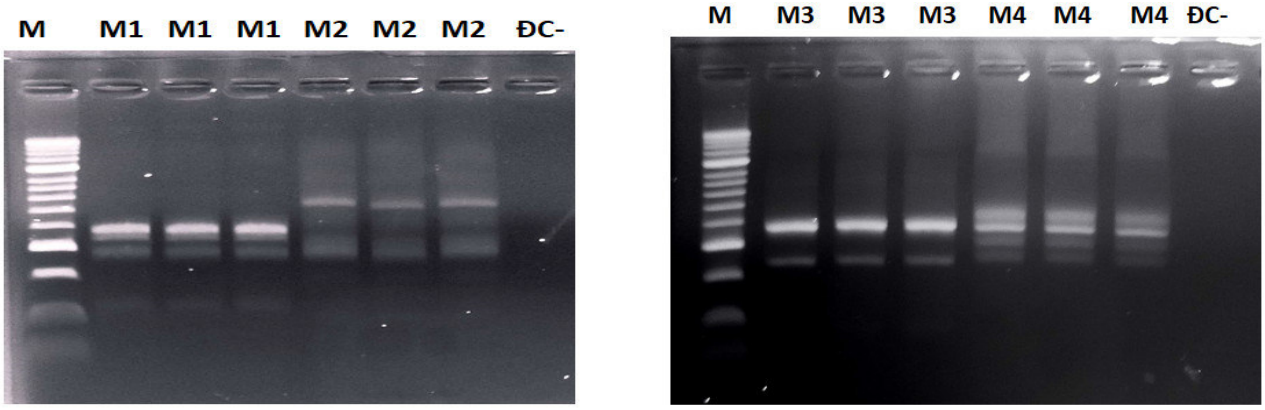

Figure 2. Gel electrophoresis of multiplex PCR product of 17 SSRs

Lane M1, M2, M3, M4 are Multiplex-PCR products of SSRs; lane ĐC: Negative samples; M: Ladder 100 bp.

The PCR was optimized with a clear and unbroken band no by-products, size corresponding to each SSR marker (Figure 1). The initial single-primer PCR optimization results showed fragments sized in the range of 200-500 bp. This result was consistent with previous studies [10], [11]. Additionally, four multiplex PCR sets of seventeen microsatellites were successfully optimized as shown in figure 2.

3.2. The polymorphisms of microsatellite/SSRs

The polymorphism of the seventeen microsatellite markers is shown in table 2.

Table 2. Polymorphism of the seventeen microsatellite markers

SSRs/Microsatellite	N	Na	Ne	Ho	He	PIC	HWE test
SSR 1	60	3	2.67	0.733	0.625	0.555	***
SSR 2	60	2	1.96	0.850	0.489	0.369	***
SSR 3	60	4	3.07	0.767	0.675	0.624	**
SSR 4	60	3	2.69	0.750	0.628	0.557	***
SSR 5	60	4	2.63	1.000	0.620	0.545	***
SSR 6	60	5	4.44	1.000	0.775	0.738	***
SSR 7	60	4	2.11	0.400	0.526	0.481	***
SSR 8	60	2	2.00	1.000	0.500	0.375	***
SSR 9	60	4	2.85	0.800	0.649	0.582	***
SSR 10	60	5	4.19	0.867	0.761	0.721	***
SSR 11	60	4	2.62	0.683	0.618	0.563	***
.SSR 12	60	3	2.57	1.000	0.611	0.533	***
SSR 13	60	2	2.00	1.000	0.500	0.375	***
SSR 14	60	5	4.31	1.000	0.768	0.731	***
SSR 15	60	3	2.64	1.000	0.622	0.550	***
SSR 16	59	5	3.69	0.627	0.729	0.686	***
SSR 17	59	5	3.78	1.000	0.735	0.691	***
Mean	60	4	2,95	0,852	0,637	0,569	

N: samples size; Na: alleles per locus; Ne: effective alleles; Ho and He: observed and expected heterozygosities; PIC: polymorphism information content;

*** Significant difference with Hardy–Weinberg equilibrium at p<0.01 level;*

**** Significant difference with Hardy–Weinberg equilibrium at p<0.001 level.*

A total of 63 alleles with sizes of 200 bp-500 bp were detected at seventeen microsatellite loci in the Phu Ninh tilapia strain (Table 2). The average number of Na and Ne were 4.0 and 2.95, respectively. Five microsatellites - SSR6, SSR10, SSR14, SSR16, and SSR17 have the highest number of alleles with the appearance of five alleles. The second highest group were SSR5, SSR7, SSR9, SSR11 with four alleles. The third group includes SSR1, SSR4, and SSR12 with three alleles. The SSR2, SSR8, and SSR13 loci appeared two alleles.

For polymorphism information content, the PIC value of each locus ranged from 0.369 to 0.738, with an average of 0.569 (Table 2). The PIC value is considered as indicator of genetic polymorphism and the degree of genetic variation. PIC>0.5 is highly polymorphic; 0.25<PIC<0.5 is a reasonable polymorphism and PIC<0.25 is a slight polymorphism [12], [13]. In

this study, thirteen loci were considered highly polymorphic (PIC > 0.5); four loci - SSR2, SSR7, SSR8, and SSR13 were considered to have a moderate degree of polymorphism. Therefore, the above thirteen loci are suitable for use in evaluating population genetic diversity and determining SSRs associated with disease resistance. Sun *et al.* (2015) reported the association between SSR markers and growth traits in Mandarin fish. The study detected 18/120 SSRs with high polymorphism (the average of PIC is 0.50) which were selected for testing correlation with growth (weight, length, and height) [14].

The observed heterozygosity (Ho) and expected heterozygosity (He) ranged from 0.400 to 1.000 and 0.489 to 0.775, respectively. The average Ho and He values were 0.852 (only SSR7 had Ho<0.5) and 0.637 (only SSR2 had Ho<0.5), respectively. In this study, the Ho was higher than the He, indicating that all loci were heterozygous. Heterozygousness within a population can be considered as a "measurement" of the genetic diversity of the population. Kotzé and Muller (1994) [15] reported that if a population is highly heterozygous for a single locus, the levels of genetic diversity at many different loci will also be high. The higher of heterozygosity parameter, the more genetic variation there is, and the opposite. In addition, comparing Ho and He can reveal the influence of foreign gene flows on the genetics of a population. Specifically, if Ho>He, the presence of foreign gene lines has a great influence on increasing heterozygosity in the population [16]. In this study, the Phu Ninh tilapia strain was the 2nd selective breeding generation (G2). The Phu Ninh tilapia strain was selected from intercrossbreeding four different strains of Nile tilapia (Genetically Improved Farmed Tilapia - GIFT strain; a GIFT-derived strain named NOVIT4; Chinese tilapia strain from ProGift Aquaculture Technology Co. Ltd. (ProGift); and a Philippine strain from Freshwater Aquaculture Center of Central Luzon State University) [17]. Therefore, many different genetic sources influenced polymorphism in the population of the Phu Ninh tilapia strain.

The results also show that all SSRs deviated from the Hardy-Weinberg equilibrium (P<0.01). In this study, it may be due to small number of samples, families (three families), and low frequency appear alleles (allele fequencies of allele of 334. in locus SSR5; allele of 231. in locus SSR7; allele of 371. in locus SSR9; allele of 352. in locus SSR10; allele of 375. in locus SSR11; result is not shown in the present study), and thus Hardy-Weinberg may be violated [18]. Crossbreeding between tilapia lines of different origins is an effective solution to help maintain allele diversity and can partially restore lost genetic diversity [19].

The polymorphism of microsatellites in this study was similar to that reported in the study of Chen *et al.* (2022) [1] and more than that reported in the study of Dong *et al.* (2008)[20]. In Nile tilapia, total alleles were identified of 62; 70, and 46 alleles in the A, B, and N2 strains, respectively. The mean number of Na, Ne, and PIC were 6.2; 3.015; and 0.575, respectively [1]. In the Chinese shrimp, the mean number of Na, and PIC were 2.5; and 0.446, respectively [20]. The present study found that 13/17 microsatellite markers had high polymorphism, except for SSR2, SSR7, and SSR13 markers.

3.3. The potential microsatellite/SSRs associated with disease resistance by *S. agalactiae*

The genotype distributions and allele frequencies of the SSRs in the hepcidin/HAMP genes in two tilapia groups (dead and alive) are shown in table 3.

There were significant differences in the SSRs genotype distributions and allele frequencies of four microsatellites - SSR3, SSR5, SSR14, and SSR15 (P<0.05) between alive and dead groups. The remaining nine SSRs had no significant differences between SSR genotypes and allele distributions (P>0.05) (Table 2). These results indicated that four microsatellite loci (SSR3, SSR5, SSR14, and SSR15) linked the hepcidin/HAMP were possibly associated with resistance to *S. agalactiae* in the Phu Ninh tilapia strain.

Table 3. The allele and genotype distributions of thirteen SSR_s in the hepcidin/HAMP genes in two groups of tilapia after challenge with *S. agalactiae*

SSRs	Gen/ID	Primer sequences (5'-3')	Motif	Size (bp)	Dyes
Multiplex I:					
SSR4	HAMP2 3'end 4.3kb (ID: 100698871)	F: CACCACTGTCAACTGGCTAATG R:GTTACCTTCTTGATACCGCAGG	$(CTAC)_7$	359	FAM
SSR5	HAMP2 3'end 4.7kb (ID: 100698871)	F: CTTTGGGTAGAGGAACACTCCA R:TGCAGGTCAATAGCAATACCAC	$(TG)_{21}$	309	HEX
SSR9	HAMP1 5'end 1.3kb (ID: 109204256)	F: GCGCTGTATAAGATTCCCGTTA R:GGAAACACAAGAGACATGAGCA	$(T)_{20}$	370	TAM
SSR14	HAMP1 reverse strand 3'end 6.2kb (ID: 100534415)	F: TCGGAATTGAGCATTAAGACCT R:GGTTCCCCATAGAACTCCTTTT	$(TG)_{22}$	245	ROX
Multiplex II:					
SSR6	HAMP1 5'end 2kb (ID: 109204280)	F: CAGTGGGTGTTTGTTCCTTACA R:TAGTAGGCTTTGTGTGCATTCC	$(GT)_{41}$	315	HEX
SSR8	HAMP1 5'end 1.3kb (ID: 109204256)	F: CCCCATAGCACTCCTTTTATTG R:TCATTTGGAGGTGTTTTCACAG	$(TG)_{22}$	322	ROX
SSR10	HAMP1 3'end 7.6kb (ID: 109204256)	F: GTTTGTAGCTTAACCCATTCGC R:TGCCTTTGTTTAGATGAACTGC	$(TG)_{10}$	364	FAM
SSR17	HAMP1 3'end 4.2kb (ID: 109204285)	F: GGTTCCCCATAGAACTCCTTTT R:TCGGAATTGAGCATTAAGACCT	$(TG)_{15}$	261	TAM
Multiplex III:					
SSR1	HAMP2 5'end 3.8kb (ID: 100698871)	F: CCACACAATCAACACACTGGTA R:TCAAACAGAAACAGGGACACAC	$(TA)_{11}$	375	HEX
SSR2	HAMP2 5'end 3.8kb (ID: 100698871)	F: GCACAGACACAGTAACACATGC R: ACTCCCTGGTACATGCTTCCTA	$(CAGG)_6$	368	FAM

SSR13	HAMP1 reverse strand 5'end 6.3kb (ID: 109204255)	F: TTAAAATGGGCTCAGGAGAAAG R:ACACACATAGATTCATCCGCAC	$(ATTC)_7$	332	ROX
SSR16	HAMP1 reverse strand intron2 (ID: 100534415)	F: GTGGGAAACACAAGAGACATGA R:CAGGGCTGAGATAGATTTTGGT	$(T)_{23}$	232	TAM
Multiplex IV:					
SSR3	HAMP2 5'end 3.8kb (ID: 100698871)	F: TAGGAAGCATGTACCAGGGAGT R: AAAATCACTCAACCGTGTCCTT	$(GT)_{13}$	348	FAM
SSR7	HAMP1 3'end 4.7kb (ID: 109204280)	F: CCACACGCACTCTCACCA R:CTGGTAGCCTTGACCCAGTTT	$(AC)_{12}$	236	HEX
SSR11	HAMP4 5'end 1kb (ID: 109204092)	F: AAGTGTCGTTCCACCCACAT R:ACAGAGTGTTCTGGCTTTCACA	$(TG)_{29}$	382	TAM
SSR12	HAMP1 reverse strand 5'end 2kb (ID: 109204255)	F: GTGCATTACAGAGTGTTCTGGC R: GTCTGGAGCCAAGTGTCGTT	$(TG)_{27}$	395	HEX
SSR15	HAMP1 reverse strand 3'end 750bp (ID: 100534415)	F: CAACAAGTGAAGCGACCATATT R:TCACACACAAGCAGGTCAATTA	$AC)_{13}$	300	ROX

Allele names (A, B, C,D, …) applied only for each locus

SSR3, SSR5, SSR14, and SSR15 are associated with the hepcidin/HAMP1 gene (SR14, SSR15) and the hepcidin/HAMP2 gene (SSR3, SSR5) in 3'-/5'- flanking region (as shown in Table 1). The 5′ flanking region has mainly functions in the regulation of gene transcription. The region contains the promoter and enhancers or other protein binding sites. The 3' flanking region often contains sequences that affect the formation of the 3' end of the Message RNA (mRNA). It may also contain enhancers or other protein binding sites [21-23]. SSRs located in the 5' flanking region of an immune-related susceptibility gene (STAT4-MS1-254), were significantly associated with sarcoidosis in humans [24]. In Nile tilapia, SSRs with (GT)n in the 5' upstream region of prolactin 1 (prl 1) were associated with differences in prl 1 gene expression and the growth response of salt-challenged fishes [23]. In this study, four microsatellites are located on chromosome 11 (LG 11) in the gene map of tilapia. The hepcidin/HAMP1 and hepcidin/HAMP2 genes were structurally similar to the hepcidin/TH1-5 and hepcidin/TH2-3 genes, containing 22 amino acids GIKCRFCCGCCTPGICGVCCRF and 26 amino acids QSHLSLCRWCCNCCRSNKGC in the protein-coding regions, respectively [2], [4]. The hepcidin/HAMP1 and hepcidin/HAMP2 genes were reported to defend against various bacterial pathogens (both gram-negative and gram-positive bacteria) in tilapia, such as *S. iniae, S. aureus, V. vulnificus* [1], [2], [3], [4]. Among 12 hepcidin/HAMP genes on LG11, 7 HAMP1, 1

HAMP2, 1 HAMP3, and 3 HAMP4 were detected [1], [10], [11], [25].

The results of our study are consent with previous studies, suggesting the relationship between SSR markers on the HAMP1 and HAMP2 genes and *Streptococcus sp.* resistance in Nile tilapia. Nhat *et al.* (2021) [26] identified three molecular markers (SSR7, SSR9, SSR16) associated with the hepcidin gene illustrating *S. inae* resistance tilapia cultured in Taiwan. In Nile tilapia, eleven disease-resistance-associated microsatellite markers (three markers in HAMP2 gene; four in HAMP1 gene; one in PGRN2 gene; two in PGRN1 gene; and one in piscidin-4 (TP4) gene) were found in tilapia strains farmed in Taiwan after challenging test with *S. inae* [1].

4. CONCLUSION

This study initially identified four potential SSRs (SSR3, SSR5, SSR14, and SSR15) linked to hepcidin/HAMP gene possibly associated with disease resistance to *S. agalactiae* in Phu Ninh tilapia strain ($P<0.05$). These potential markers can be applied as) MAS in the selection of tilapia associated with Streptococosis resistance caused by *S. agalactiae* in Vietnam.

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CURRENT STATUS AND TECHNICAL SOLUTIONS NEEDED TO BE IMPLEMENTED FOR EFFICIENT AND SUSTAINABLE SNOUT OTTER CIAM (*Lutraria rhynchaena*) CULTURING

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ABSTRACT

The article presents the results of the current status of Snout Otter Clam farming techniques in Quang Ninh, Hai Phong, and Khanh Hoa through questionnaires and information collection in order to determine the main technical parameters in the current Snout Otter Clam culture and some technical solutions that need to be implemented. This is the first facility to serve the construction of a highly efficient and sustainable commercial Snout Otter Clam farming technology process. Regarding the current status of farming techniques, the total area of Snout Otter Clam farming in the three surveyed provinces is estimated at 299 hectares, and farmers mainly apply the cage culture placed in the tidal flat on about 214 hectares. The stocking size is from 1 - 3 cm/individual with a density of 25 - 55 fish/cage, and the time of stocking is from March to April every year. The culture period is 10-14 months per crop, the harvested Snout Otter Clam size is 25 - 60 g/individual, the survival rate is 25 - 60%, and the yield is 355.37 - 470.84 g/cage/crop. Regarding the epidemic situation, there was no outbreak of disease. However, it was still recorded that diseased Snout Otter Clam appeared sporadically during the culture period. The characteristic presentation described is similar to that of Virus-like particles (VLPs). Snout Otter Clam are diseased at seed stage (size from 2 - 3 cm) and commercial stage (size from 20 - 35 g/head). The research results have provided the current status of Snout Otter Clam farming and suggested the main solutions that need to be taken to develop an effective and sustainable Snout Otter Clam culture.

Keywords: *Current status, technical solutions, farming techniques, snout Otter Clam.*

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1. INTRODUCTION

Snout Otter Clam is one of the molluscs with the advantages of high economic value, suitable habitat conditions, and easy-to-apply farming techniques. Snout Otter Clamproducts are easy to consume in the market because Snout Otter Clam meat is fragrant, delicious, and rich in protein. In particular, Snout Otter Clam meat also contains 18 kinds of amino acids, of which some are not substituted and have a high content [1]. Snout Otter Clam are also widely cultivated in Southeast Asia and the southeastern coastal areas of China and have become an important fishery species in these regions [2]. This species contributed an important part to the production of 16.1 million tons of bivalve molluscs in 2016, accounting for 21.4% of global aquaculture production [3] and most of the mollusc production. Marine bivalves (89%) come from aquaculture, with a total economic value of $20.6 billion per year [4].

Snout Otter Clam has become one of the key species of mollusk farming in the past few years in Vietnam. Snout Otter Clam is mainly distributed in the northern region of Ha Long Bay (from Cat Ba Island, Hai Phong, to Van Don, Quang Ninh) and

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in the central coastal area (Nha Trang, Khanh Hoa), Snout Otter Clam farming is mainly concentrated in Quang Ninh, Hai Phong, and Khanh Hoa provinces. There were times when geoduck farming developed rapidly, with thousands of farming households in these areas [5]. Due to the serious disease situation in Snout Otter Clam, the Snout Otter Clam farming industry has decreased sharply in terms of farming area and production. However, it is undeniable that Snout Otter Clam is still one of the objects of great economic value among today's farmed mollusks. In order to effectively restore and develop Snout Otter Clam farming, it is necessary to develop a new farming technique in the direction of safety, sustainability, and an increase in output and value of goods. From these issues, the objective of this study is to survey and evaluate the current technical status of Snout Otter Clam farming in Quang Ninh, Hai Phong, and Khanh Hoa the main Snout Otter Clam farming provinces. Thereby assessing the limitations that need to be overcome and providing technical solutions that need to be implemented. This is a very important first step to solve, serving to build a highly efficient and sustainable commercial Snout Otter Clam (*Lutraria rhynchaena*) culture process from which it can be applied in production practice.

2. RESEARCH METHODOLOGY

2.1. Time, place, number of survey samples

- Execution time: From April 2021 to November 2021.

- Survey location: Quang Ninh (in Van Don, Cam Pha), Hai Phong (in Cat Ba), Khanh Hoa (in Nha Trang).

- Number of questionnaire samples: The total number of samples to collect information was 270, evenly distributed among the 3 survey provinces according to Yamane's formula (1967) [6]. Each province has 90 samples. In each surveyed province, 10 questionnaires were allocated to interview managers at the fisheries department and 80 questionnaires to interview Snout Otter Clam farming households. In the province, the survey identified districts with Snout Otter Clam farming activities; accordingly, Quang Ninh concentrated on Van Don, Hai Phong concentrated on Cat Hai, and Khanh Hoa concentrated on Nha Trang. In each district, the samples are equally distributed to communes with current Snout Otter Clam farming activities, with the following numbers: Van Do (Ban Sen: 20 samples; Doan Ket: 20 samples; Ha Long: 20 samples; Cai Rong: 20 samples); Cat Hai (Cat Ba: 80 samples); Nha Trang (Vinh Luong: 80 samples). After calculation, the samples are randomly distributed to the corresponding farming areas. For communes with fewer farming households than the number of allocation votes, a survey of the entire number of farming households was conducted.

2.2. Building the survey form

Building four types of questionnaires to collect information, specifically as follows:

- Type 1: The questionnaire collects information from managers of the provincial fisheries departments on the current technical status of Snout Otter Clam farming. Each province surveyed five questionnaires, focusing on key information such as the number of farming households, total farming area, form of Snout Otter Clam farming, advantages and disadvantages of current Snout Otter Clam farming, and technical solutions that need to be implemented.

- Type 2: The questionnaire collects information from Snout Otter Clam farming households on the current technical status of Snout Otter Clam farming. Each province surveyed 40 questionnaires, focusing on key information including: water environment conditions in the farming area; farming form; density; stocking stock size; bottom material; density of cages; survival rate; harvest size; yield; and economic efficiency.

- Type 3: The questionnaire collects information from managers of the provincial fisheries departments on the current situation of Snout Otter Clam diseases. Each province surveyed five questionnaires, focusing on the

advantages and disadvantages of Snout Otter Clam disease management and the main technical solutions that need to be implemented to minimize the disease.

- Type 4: The questionnaire collects information from Snout Otter Clam farming households on the current situation of Snout Otter Clam diseases. Each province surveyed 40 questionnaires, focusing on key information including: diseased Snout Otter Clam and disease manifestations; time, season, and stage of disease occurrence. Subjective statements about the cause of the disease, measures that have been applied to prevent disease, and the effectiveness of the measures.

2.3. Methods of investigation and data collection

Using standardized questionnaires (survey form) and the Rapid Rural Appraisal (RRA) method [7], [8] directly interviewed Snout Otter Clam farmers, officials managed at the Fisheries Sub-Departments of Quang Ninh, Hai Phong, and Khanh Hoa provinces.

Primary data were collected through interviews and standardized questionnaires. For some water environment factors in the farming area (temperature, salinity, pH, and DO), they were collected by interview and combined with direct measurement. Temperature was measured with a mercury thermometer graduated in 0.1°C, salinity was measured with a refractometer with 1ppt graduations, pH was measured with a hand-held pH meter, and DO was measured with a Kit Sera.

Secondary data were collected from research projects on Snout Otter Clam that have been accepted, from published articles, reports on disease situations in Snout Otter Clam and mollusc farming, and directive documents from management agencies at all levels.

2.4. Data processing methods

The data is processed by descriptive statistics through calculating the average (Mean), minimum (Min), maximum (Max), and analytical methods variance ANOVA on Minitab 16 software.

3. RESULTS AND DISCUSSION

3.1. Technical status of Snout Otter Clam culturing

3.1.1. Water quality in the farming area

Water quality in the farming area plays an important role in commercial Snout Otter Clam farming. Environmental factors affecting the growth and survival rate of Snout Otter Clam, such as hydrological, hydrochemical, and aquatic factors. In which these factors are interrelated and influence each other. The physicochemical factors such as salinity, clarity, temperature, and pH affect the growth and development of wild Snout Otter Clam as well as commercial Snout Otter Clam culture, in which the most important factors are salinity and turbidity of the water. The results of the survey and collection of some water environmental factors in Snout Otter Clam farming areas in three provinces are shown in table 1.

Table 1. Some water environmental favameters in Snout Otter Clamfarming areas

Survey province	Temperature (°C)	Salinity (‰)	pH	DO (mg/L)
Quang Ninh	15 - 30	26 - 30	8.0 - 8.5	5.0 - 6.5
Hai Phong	15 - 30	26 - 30	7.5 - 8.5	5.0 - 6.5
Khanh Hoa	22 - 30.5	28 - 32	8.0 - 8.5	5.0 - 6.0

The results show that some water environmental favameters in Snout Otter Clam farming areas in Quang Ninh, Hai Phong, and Khanh Hoa are relatively similar. The water temperature fluctuates between 15°C and 30°C, with an average of 26.5°C (in Quang Ninh and Hai Phong) and 27.5°C (in Khanh Hoa). The water temperature in the farming area has little variation during the day; the difference between morning and afternoon is about 1 - 2°C. However, water temperature has a clear seasonal variation; the lowest temperature can be observed (15°C in

Quang Ninh and Hai Phong; 22°C in Khanh Hoa) on some days in the months from November to January. The highest temperature (30°C in Quang Ninh and Hai Phong; 30.5°C in Khanh Hoa) is observed in the months from July to September every year. According to Luca and Doan Xuan Nam (2012), the tolerance threshold for Snout Otter Clamis 12 - 37°C, and the optimal temperature for growth ranges from 24 -28°C [9].

The salinity of the water environment measured at the time of the survey in Quang Ninh and Hai Phong ranges from 26‰ to 30‰. Meanwhile, the salinity in Snout Otter Clam farming areas in Khanh Hoa often fluctuates between 28‰ and 32‰. This is a suitable range of salinity for the growth and development of Snout Otter Clam [10].

pH values ranged from 8.5 - 8.5 (Quang Ninh, Khanh Hoa) and 7.5 - 8.5 (Hai Phong). This pH range is considered suitable for marine aquaculture species in general and Snout Otter Clam in particular [11].

Dissolved oxygen content ranged from 5.0 - 6.5 mg/l. The appropriate dissolved oxygen content for Snout Otter Clam is 5 mg/l or more, and the allowable oxygen level in the Snout Otter Clamhabitat is 4 mg/l [12].

In general, in the surveyed Snout Otter Clam farming areas, temperature, salinity, pH, and dissolved oxygen were suitable for Snout Otter Clam growth.

3.1.2. Current status of farming areas and farming methods

Through the collection of available information and survey results in Quang Ninh, Hai Phong, and Khanh Hoa, it is shown that currently, Snout Otter Clam are farmed in a very limited areas, and production as well as farming area have decreased markedly compared to that of 5 - 10 years ago. The province with the largest Snout Otter Clam farming area in the three surveyed provinces is Quang Ninh, with about 152 farming households and an estimated total farming area of 209 hectares. Hai Phong is the province with at least 50 farming households and an estimated area of 40 ha (Table 2).

Table 2. General Status of Snout Otter Clam Farming

Survey provinces	Number of farming households (household)	Farming area (ha)	Farming form		
			Cages hanging on raft (ha)	*Cage placed on the tidal flat (ha)*	*Drop directly into the tidal flat (ha)*
Quang Ninh	152	209	60	149	-
Hai Phong	50	40	15	25	-
Khanh Hoa	60	50	18	40	2
Total	262	299	93	214	2

Snout Otter Clam farming methods include cages hanging on raft, cages placed on the tidal flat, and dropping directly into the tidal flat. Currently, only a few households in Khanh Hoa still keep farming in dropping directly into the tidal flat form, with an estimated farming area of about 2 hectares. Farmers mainly farm in the form of cages placed on the tidal flat, with a total farming area of about 214 ha (Table 2). Snout Otter Clam farming cages are hung below the tide with the lowest depth at low tide of 1.5 - 2 m (for the cages hanging on rafts) and placed relatively flat in the intertidal areas (for the cages placed on the tidal flat), with the lowest depth at low tide of 0.5-1.5 m, the bottom is sand mixed with mollusk shells and coral debris. The current selection of Snout Otter Clam farming methods is completely consistent with reality in Quang Ninh, Hai Phong, and Khanh Hoa. For the form of drop directly into the tidal flat, the area of yards capable of natural stocking is not much, the management and the ability to recover commercial Snout Otter Clam are difficult for the tidal flats with great depth, and moreover, direct free-range farming has a very low survival

rate, so drop directly into the tidal flat is almost no longer applied. For the form of cages hanging on raft, the initial investment is quite hight, and it is necessary to have a farming location that meets the requirements of depth and flow and is less affected by wind and storms to place rafts. This is not something many households can do. Therefore, the main farming method applied in Quang Ninh, Hai Phong, and Khanh Hoa is still cage placed on the tidal flat.

3.1.3. The current status of some key technical parameters in Snout Otter Clam farming

The results of the survey on some current Snout Otter Clam farming specifications in Quang Ninh, Hai Phong, and Khanh Hoa are shown in table 3.

Table 3. Current Snout Otter Clam farming specifications

Specifications	Quang Ninh	Hai Phong	Khanh Hoa
Size of seed (cm/individual)	2.13 ± 0.13 1 - 3	2.10 ± 0.16 1 - 3	2.18 ± 0,15 1 - 3
Stocking density (individuals per cage)	32.98 ± 0.77 25-50	33.30 ± 1.06 25-55	33.20 ± 0.84 25-50
Density of cages (cages/m^2)	3.45 ± 0.14^a 2-4	2.65 ± 0.15^b 2-4	2.55 ± 0.14^b 2-4
Breeding period (months)	10.88 ± 0.13 10-12	10.95 ± 0.17 10-14	11.00 ± 0.21 10-14
Harvest size (g/individual)	31.88 ± 1.08 25-57	32.85 ± 1.02 25-60	34.63 ± 1.03 25-60
Survival rate (%)	33.25 ± 1.25^b 25-55	40.85 ± 1.58^a 30-60	40.67 ± 1.69^a 25-60
The yield (g/cage/crop)	355.37 ± 19.39^b	454.56 ± 32.22^a	470.84 ± 27.55^a

The Size of seed in Quang Ninh, Hai Phong, and Khanh Hoa were relatively uniform, ranging from 1 - 3 cm, with an average of 2.13 cm (Quang Ninh); 2.10 cm (Hai Phong); and 2.18 cm (Khanh Hoa). There was no significant difference ($P > 0.05$) in seed size at stocking in the three surveyed provinces.

Stocking density ranged from 25 to 55 individuals per cage (cage size is 60 cm x 45 cm x 35 cm) depending on stocking size, equivalent to about 100 to 200 individuals/m^2. Stocking density was also relatively uniform in the surveyed provinces ($p > 0.05$). In Quang Ninh and Khanh Hoa, stocking densities range from 25 - 50 individuals/cage, with an average of 32.98 individuals/cage (Quang Ninh) and 33.20 individuals/cage (Khanh Hoa). In Hai Phong, stocking density ranged from 25 - 55 individual/cage, with an average of 33.30 individuals/cage.

The density of cages was from 2 - 4 cages/m^2 in yards or hanging rafts. The average density of cages was different among the surveyed provinces ($p < 0.05$); the density of cages was highest in Quang Ninh (3.45 cages/m^2), and there was no difference between the density of cages in Hai Phong (2.65 cages/m^2) and Khanh Hoa (2.55 cages/m^2).

The period of commercial Snout Otter Clam farming lasts from 10 to 14 months, depending on the seed size at stocking. The time of stocking is from March to April of the solar calendar, and harvesting is from January to February of the following year. The mean culture period in Khanh Hoa (11 months) tended to be longer than in Quang Ninh (10.8 months) and Hai Phong (10.9

months), but this difference was not statistically significant ($p > 0.05$).

The survival rate of Snout Otter Clam is often unstable, ranging from 25-60%. There was a significant difference in the survival rate of Snout Otter Clam in the three surveyed provinces ($p < 0.05$). The lowest survival rate in Quang Ninh is 33.25%, followed by Khanh Hoa at 40.67% and Hai Phong at 40.85%. There was no difference in survival rates between Khanh Hoa and Hai Phong. The results showed that the survival rate tended to decrease when the density of cages was high. In Quang Ninh, the density of cages was significantly higher than in Hai Phong and Khanh Hoa, where the survival rate was lower.

Snout Otter Clam production at harvest was also found to be different among the three surveyed provinces ($p < 0.05$). The lowest yield was obtained in Quang Ninh with 355.37 g/cage, the highest in Khanh Hoa (470.84 g/cage) and Hai Phong (454.56 g/cage). This result also shows that there is a tendency for the density of cages and survival rate to influence yield at harvest. When the density of cages is high, the survival rate is low, and the yield is also lower.

3.1.4. Current status of Snout Otter Clam disease

The results of the survey showed that the disease in Snout Otter Clam is now much more limited than in previous years; there are no outbreaks of diseases because Snout Otter Clam farming households have decreased significantly in both quantity and scale. However, in the three surveyed provinces, Snout Otter Clam diseases were still recorded, appearing sporadically during the farming period. The initial subjective judgment of the households may be due to the quality of the input breed. The characteristic presentation is described as similar to that of Virus-like particles (VLPs). Diseased Snout Otter Clamat seed stage (size from 2 - 3 cm) and commercial stage (size from 20 - 35 g/individual. For Snout Otter Clam seed, the disease period is usually from May to July every year. In the commercial stage, the disease period is usually from November to January of the following year (Table 4).

Table 4. Disease status in Snout Otter Clam

Status quo	Quang Ninh	Hai Phong	Khanh Hoa
Time of disease appearance	May-July; December - January of the following year	May-July; December -January of the following year	May-July; December - January of the following year
Disease stage and size of Snout Otter Clam	Seed stage (2 - 3 cm); Commercial stage (20 -30 g/ind)	Seed stage (2 - 3 cm); Commercial stage (30 - 35 g/ind)	Seed stage (2 – 3 cm); Commercial stage (20 - 35 g/ind)
Disease manifestation	The faucet is swollen and surrounded by white fluid.	The faucet is swollen and surrounded by white fluid.	The faucet is swollen and surrounded by white fluid.
Measures taken to prevent the disease	Select suitable breeding sites; select breeds according to color, uniform size, and no abnormalities; Stocking at the right time.	Select suitable breeding sites; select breeds according to color, uniform size, and no abnormalities; Stocking at the right time.	Select suitable breeding sites; select breeds according to color, uniform size, and no abnormalities; Stocking at the right time.

The survey results also show that, now that Snout Otter Clam farming has decreased sharply, many households have switched from Snout Otter Clam farming to Pacific oyster farming, clam farming, etc. Therefore, Snout Otter Clam farming households currently mainly apply measures for mollusc farming in general to limit the occurrence of diseases, such as: selecting suitable farming sites; selecting breeding stock through the sense of color, size, and dimorphism; Stocking at the

right time; and checking Snout Otter Clam growth periodically.

3.2. Technical solutions to be implemented for effective and sustainable Snout Otter Clam farming

The evaluation of the survey results on the current status of Snout Otter Clam farming shows that Snout Otter Clam farming in Vietnam in general has not developed commensurate with its potential (in terms of water surface area, available food sources, etc.). Previously, in Snout Otter Clam farming technology, only different farming methods were introduced, but there was no specific farming protocol for different farming methods. Tran The Muu (2011) [10] initially successfully developed the commercial Snout Otter Clam farming protocol. Until now, Snout Otter Clam farming techniques have still applied this protocol. However, the protocol still has limited technical parameters such as small seed size, low stocking density, long farming time, low survival rate, and low productivity. The protocol has also not been mentioned regarding cage density, bottom substance in commercial Snout Otter Clam farming, and care mode. A solution to these specifications is needed for efficient and sustainable snout otter clam farming. The technical specifications are limited and need to be implemented, specifically:

Seed quality: The quality of seed is very important in Snout Otter Clam farming; the source of seed is a factor that can affect the whole farming process. The 2017 Fisheries Law has been implemented, which requires that the seed be quality quarantined before being sold. However, the results of the survey on the quality of seed showed that most of the Snout Otter Clam seed from the hatchery did not go through quarantine; the quality standards of the seed were only evaluated through the senses and based on the trust between the buyers and the seller. This leads to an uncontrollable source of disease from seed and a high risk of disease outbreaks. Dang Thi Lua (2018) have identified the VLPs agent at the stage of Snout Otter Clam rearing in hatcheries and confirmed that Snout Otter Clam larvae are the main source of VLPs pathogens [5]. Therefore, when selecting seeds, it is necessary to check for VLPs to ensure that the seed are free from VLPs. At the same time, select seeds with clear origins from reputable seed suppliers, bright natural colors, uniform sizes, healthy, unbroken shells, and a low malformation rate.

Size of stock, stocking density, and time of rearing: the current stocking size is mainly small, the size fluctuates about 2 cm, and the stocking density is over 30 individuals per cage (120 individuals/m^2). After 11 months of rearing, the commercial size is only 30 - 35 g/cage, and the yield is from 0.35 to 0.5 kg/cage. This seed size and stocking density are consistent with the procedure of Tran The Muu (2011) [10], which states that in seed selection, the appropriate initial stocking size is grade 2 seed (shell length 1.5 - 3 cm) with a stocking density of 25 - 30 individuals per cage. However, with such stocking density,the nutritional potential of the culture area has not been fully promoted, leading to low productivity. At the same time, small stocking sizes are also associated with enemy attacks and often cause high mortality. Research by MacKenzie Jr. (1977) on the stocking size of the clam *Mercenaria mercenaria* showed that stocking sizes < 20 cm are often attacked by many types of snails, crabs, and starfish [13]. For shrimp, the stocking size for commercial farming is over 2 cm [14]. According to the report of the Center for Agriculture and Fisheries Extension in Phu Yen, since 2010, the commercial Snout Otter Clam farming model has been implemented with a stocking size of 3 cm. Stocking density is 50 to 60 individuals per After nine months of stocking commercial Snout Otter Clam, it reached 45.4 – 50 g/individual. Therefore, in order for Snout Otter Clam farming to be more effective in improving survival rates, productivity, and reducing farming time, it is necessary to have studies on testing large stocking sizes of 2 cm or more and stocking density of over 30 individuals per cage.

Density of cages: Currently, there is no research on the appropriate density of commercial cages per unit of farming area. Farmers mainly put cages next to each other, affecting the water

exchange between the cage and the environment. At the same time, the survey results show that there is a tendency for Snout Otter Clam survival to decrease when the density of cages is above 3 cages/m^2. Therefore, it is necessary to have a trial to evaluate the effect of cage density on the survival rate of Snout Otter Clam. This can be a technical solution to improve survival rates, thereby improving productivity effectively and sustainably.

Care mode: in the process of raising Snout Otter Clam, most farmers do not pay attention to the cleaning and care of cages and are very limited in the frequency of inspection, cage cleaning, and bottom replacement. At the same time, current farming techniques have not mentioned the frequency of cleaning and care of the cages as being most effective. In a culture cycle with a long rearing time, the cage is not cleaned and cared for, which is also one of the causes of disease in Snout Otter Clam, reducing their survival rate [12]. In order for the Snout Otter Clam to grow well, have a high survival rate, and preveut disease, one of the solutions that needs to be implemented is to increase the frequency of cleaning and caring for the cages by periodically cleaning the cages, picking up all kinds of waste, washing sand, and adding sand to the cages if a loss of sand is found. The frequency of cage care also needs to be tested for the most effective Snout Otter Clam farming techniques.

Bottom substance in Snout Otter Clamculture: in the current commercial Snout Otter Clam farming process, not to mention the effect of bottom substance on commercial Snout Otter Clam, only studies on bottom substance have been done on the seed stage [10]. In the recent period, one of the causes of mass Snout Otter Clam mortality was also partly due to bottom degradation [5]. Bottom matter affects the growth and development of Snout Otter Clam, so it is necessary to study the composition of the benthic substance (sand and mollusk shell) for Snout Otter Clamand test it.

4. CONCLUSION

- The estimated total area of Snout Otter Clam farming in Quang Ninh, Hai Phong and Khanh Hoa province is 299 hectares, of which 214 hectares are raised in cages placed on the tidal flat.

- In the three provinces, there are currently about 262 Snout Otter Clam farming households. Seed sizes at stocking are from 1-3cm/individuals, stocking densities ranging from 25 - 55 shrimp/cage, time of stocking from March to April, and harvesting between January and February of the following year. The farming period is from 10 to 14 months, the size of commercial Snout Otter Clam is from 25 to 60 g/individual, the survival rate is from 25 to 60%, and the yield is from 355.37 to 470.84 g/cage (1,316 - 1,744 g/m^2).

- No outbreak of disease was found; however, there was still a phenomenon of sporadic disease in the rearing period. The initial subjective assessment may be due to the quality of seed. The characteristic presentation described is similar to that of VLPs. Snout Otter Clam were infected at the seed stage (size from 2 - 3 cm) and the commercial stage (size from 20 - 35 g/individual).

- Identified the main technical parameters that are still limited and need to be improved in order to raise the Snout Otter Clam effectively and sustainably, including stocking size, stocking density, cage density, bottom rate, and care regime. To improve these specifications, it is necessary to have experimental studies on stocking sizes above 2cm, experimenting with stocking density of over 40 findividuals per cage, cage density of less than 3 cages per m^2, testing bottom material for rearing calves, and management.

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QUALITY OF FISH OIL EXTRACTED FROM BARRAMUNDI BY-PRODUCT BY ENZYMATIC HYDROLYSIS METHOD

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ABSTRACT

Study on the fish oil extraction from barramundi by-product by enzymatic hydrolysis method was carried out. The parameters of hydrolysis process, oil recovery and chemical quality including free fatty acid content, acid value, peroxide value, iodine value and saponification value of fish oil were determined. The study results showed that a considerable amount of oil can be extracted from barramundi by-product. The optimal parameters of enzymatic hydrolysis process for recovering the fish oil were the water/material ratio of 50%, enzyme/material ratio of 0.3%, hydrolysis temperature of 60^0C and hydrolysis time of 2 hours. The fish oil obtained from hydrolysis of barramundi by - product in optimum conditions was rich in omega-3 fatty acids, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The major fatty acids in barramundi by-product oil were palmitic acid, oleic acid and docosahexaenoic acid. This study suggested that the barramundi by-product generated from fish processing industry can be utilized as a good source for fish oil recovery. Barramundi by-product oil can be used in aquaculture feed or considered as a valuable source for human consumption.

Keywords: *Barramundi by - product, enzymatic hydrolysis, fish oil extraction, quality of oil.*

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1. INTRODUCTION

Barramundi (*Lates calcarifer*) is one of the most favorite seafood in many countries of the world. Large quantities of barramundi were processed as frozen products, and in consequence a large amount of by-products (Head, skeleton, viscera, skin, trimmings, fins) was generated. Barramundi is a rich source of lipids. There is a great potential in marine bioprocess industry to convert and utilize this by -product into value - added food ingredients. One possible way to use the barramundi by-products is to extract the fish oil for the production of aquaculture feed.

The common methods used for extraction of fish oil consist of chemical extraction, cooking and pressing and enzymatic hydrolysis. The chemical extraction of fish oil is complicated, where the solvents used for extraction have to be separated requiring excess energy. The fish oil has been produced by cooking, pressing followed by centrifugal separation. This traditional method is very harsh on lipids and can lead to faster degradation of lipids. Enzymatic hydrolysis method used for oil extraction has many advantages, such as the mild hydrolysis conditions, low energy requirement, no use of solvent. The low hydrolysis temperatures minimize the oxidation of polyunsaturated fatty acids. Thus, the enzymatic extraction of oil from barramundi by-product was used in this study.

Enzymatic tissue disruption may be a valid alternative technique for releasing natural lipids from fish. During the enzymatic hydrolysis, the combination between lipid and protein was break down, which lead to fish oil release much easier from fish by-product [1]. Enzymatic hydrolysis was used to extract the fish oil using commercial proteases. Several enzymes such as Alcalase, Neutrase, Protamex and Flavourzyme can be used to extract fish oil. However, Alcalase was the best

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enzyme for the extraction of oil from fish and fish by-product [2], [3]. Therefore, in this study, Alcalase was chosen for the extraction of oil from barramundi by-product.

In the process of enzymatic hydrolysis, the factors such as water to material ratio, enzyme to material ratio, hydrolysis temperature and time influence fish oil extraction. The purpose of this study was to determine the optimal hydrolysis conditions for maximum oil recovery from barramundi by-product using Alcalase and to value the chemical quality of fish oil with various criteria, including free fatty acid, acid value, peroxide value, iodine value, saponification value and fatty acid composition.

2. MATERIALS AND METHODS

2.1. Materials

Barramundi by-product including head and skeleton of fish were provided by a seafood processing company in Nha Trang, Vietnam. After filleting at the company, barramundi by - product were stored with ice at 0 - 4^0C and transported immediately to the laboratory. The barramundi by - product were minced using a grinder (5 mm plate size), and put into the plastic bags. These bags were vacuum packaged, frozen and kept at -18 ± 2^0C until use for the hydrolysis.

2.2. Enzyme

Alcalase is a proteolytic enzyme produced by submerged fermentation of a selected strain of *Bacillus licheniformis*. Alcalase (Endopeptidase, activity 2.4 AU/g) was produced by Novozymes (Bagsvaerd, Denmark). The optimal working conditions for Alcalase are temperatures between 55 and 70^{0}C and pH between 6.5 and 8.5.

2.3. Determination of optimal hydrolysis conditions for oil extraction from barramundi by - product

2.3.1. Determination of optimal water/material ratio

In order to determine the optimal water/material ratio for oil recovery, the minced barramundi by - product were hydrolyzed by using 0.1% Alcalase in 1 hour at temperature of 55^{0}C and natural pH of material (6.5) with water/material ratios of 0%, 25%, 50%, 75% and 100%. After hydrolysis, the enzyme was inhibited by heat treatment at 85^{0}C for 15 minutes in a water bath. Then, the mixture was filtered through a mesh to remove the solid fraction (bones). The filtrate was centrifuged at 7000 rpm for 30 minutes. After centrifugation, the following four fractions were formed: The oil fraction on the top, the emulsion and the liquid protein hydrolysate in the middle and the sludge on the bottom. The oil fraction was recovered, then weighed to calculate the percentage of recovered oil. From results obtained, the optimal water/material ratio was selected.

2.3.2. Determination of optimal enzyme/ material ratio

With the optimal water/material ratio identified and hydrolysis conditions as above (In 1 hour at temperature of 55^{0}C and pH of 6.5), the minced barramundi by - product were hydrolyzed with 0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% Alcalase. After hydrolysis, the same steps as above were carried out. The optimal enzyme/material ratio was selected.

2.3.3. Determination of optimal hydrolysis temperature

With the optimal water/material and enzyme/material ratios identified, the minced barramundi by - product were hydrolyzed in 1 hour at pH 6.5 and temperature of 50^{0}C, 55^{0}C, 60^{0}C, 65^{0}C, 70^{0}C. After hydrolysis, the same steps as above were carried out. The optimal hydrolysis temperature was selected.

2.3.4. Determination of optimal hydrolysis time

With the optimal water/material and enzyme/material ratios identified, the minced barramundi by - product were hydrolyzed at pH 6.5 and optimal hydrolysis temperature identified in 1 hour, 2 hours, 3 hours, 4 hours and 5 hours. After hydrolysis, the same steps as above were carried out. The optimal hydrolysis time was selected.

2.4. Chemical analyses

Lipid content was determined according to the method of Folch *et al.* (1957) [4]. The free fatty

acid content, acid value, peroxide value, iodine value, saponification value were determined according to American Oil Chemists' Society AOCS (1997) [5]. Fatty acid composition in the oil was determined by gas chromatography according to Noriega - Rodríguez *et al.* (2009) [6].

2.5. Oil recovery

The fish oil was obtained as the top layer during the extraction process, and was removed with a pipette and weighed using a digital balance. The weight was used to calculate the percentage of recovered oil. The percentage of oil recovery from raw material was defined as follows:

Oil recovery (%) = (Weight of oil recovered/ total weight of lipid in raw material) x 100

2.6. Statistical analysis

The experiments were carried out in triplicates. The data obtained were subjected to one way analysis of variance (ANOVA), followed by the Duncan's multiple range test to determine the significant difference between samples at $p<0.05$ level using the SPSS 20 programme.

3. RESULTS AND DISCUSSION

3.1. Determination of optimal hydrolysis conditions for oil extraction from barramundi by - product

3.1.1. Determination of optimal water/material ratio

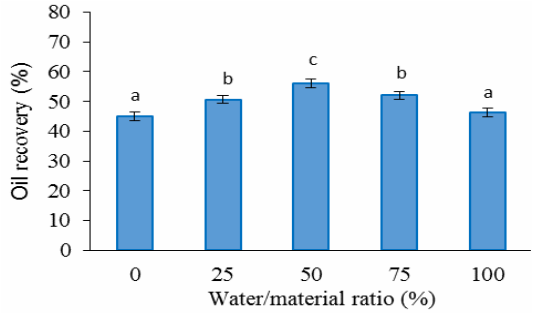

Figure 1. The influence of water/material ratio on the oil recovery from barramundi by - product. Mean values with different superscript letter are significantly different ($p<0.05$)

The influence of water/material ratio on the oil recovery from barramundi by-product is shown in figure 1.

The study result showed that the oil recovery from hydrolysis of barramundi by - product reached the highest value (56.17%) with water/material ratio of 50%. The oil recovery reduced with the increase in water/material ratio from 50 to 100%. This result was in accordance with that reported by Mbatia *et al.* (2010) who also showed that an increase in water/material ratio during the hydrolysis resulted in a decrease in oil yield [7]. Decrease in oil yield with increasing water/material ratio during the hydrolysis may be due to emulsion formation [7]. Daukšas *et al.* (2005) reported that the lipid recovery in the oil fraction after hydrolysis of different by - products ranged from 36.4% to 82.8% [8]. The previous studies indicated that the oil liberation was depended on the raw material and hydrolysis conditions [7], [8]. The study has indicated that the enzymatic hydrolysis using Alcalase with water/material ratio of 50% has brought the highest percentage of oil recovery. Therefore, the water/material ratio of 50% was optimal for oil recovery from barramundi by - products.

3.1.2. Determination of optimal enzyme/ material ratio

During the enzymatic extraction of oil from the barramundi by - product with Alcalase, enzyme/material ratio plays an important role in the recovery of oil. Figure 2 indicate the influence of enzyme/material ratio on the release of oil.

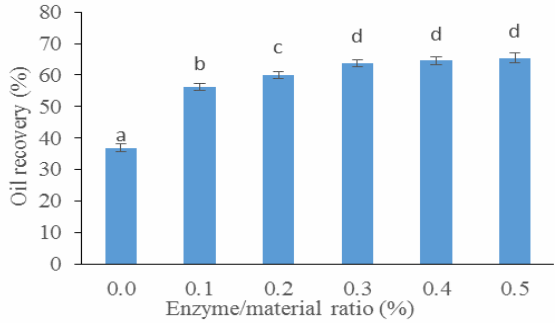

Figure 2. The influence of enzyme/material ratio on the oil recovery from barramundi by - product. Mean values with different superscript letter are significantly different ($p<0.05$)

The results demonstrated that increasing the enzyme/material ratio increased the oil recovery from barramundi by-product. The oil recovery

increased strongly from 36.85% to 63.65% with the increase of the enzyme/material ratio from 0% to 0.3%. As the enzyme concentration increases, the rate of hydrolysis increases, which leads to the increasing of oil recovery. However, there were no significant differences in oil recovery among the samples with the enzyme/material ratios of 0.3%, 0.4% and 0.5%. Mbatia *et al.* (2010) stated that maximum oil recovery from salmon head was achieved when 0.5% Bromelain was used, and showed that a higher enzyme/material ratio did not result in the further increase in oil recovery [7].

According to the results in this study, the highest oil recovery was obtained with enzyme/material ratio of 0.3%. The enzyme/material ratio above 0.3% did not improve the oil recovery. Therefore, the enzyme/material ratio of 0.3% was optimal for the oil extraction.

3.1.3. Determination of optimal hydrolysis temperature

The influence of hydrolysis temperature on the oil recovery from barramundi by-product is demonstrated in figure 3.

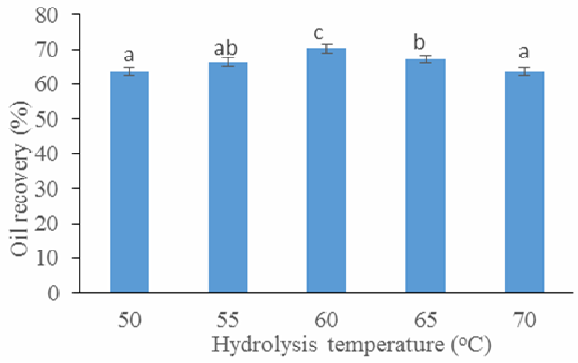

Figure 3. The influence of hydrolysis temperature on the oil recovery from barramundi by - product. Mean values with different superscript letter are significantly different (p<0.05)

The results indicated that the hydrolysis temperature had a significant effect on the oil recovery. Increasing the hydrolysis temperature increased the oil recovery from barramundi by-product. The oil recovery increased sharply from 63.60% to 70.29% with the hydrolysis temperatures in a range of 50 - 60^{0}C. The highest oil recovery was achieved at 60^{0}C. However, with the hydrolysis temperature of 65^{0}C and 70^{0}C, the oil recovery from barramundi by-product decreased in 67.07% and 63.72% respectively. This may be due to decreasing the activity of enzyme at the high temperature (65^{0}C and 70^{0}C). The results showed that the optimal temperature for oil extraction from barramundi by - product was 60^{0}C.

3.1.4. Determination of optimal hydrolysis time

The influence of hydrolysis time on the oil recovery from barramundi by-product is shown in figure 4.

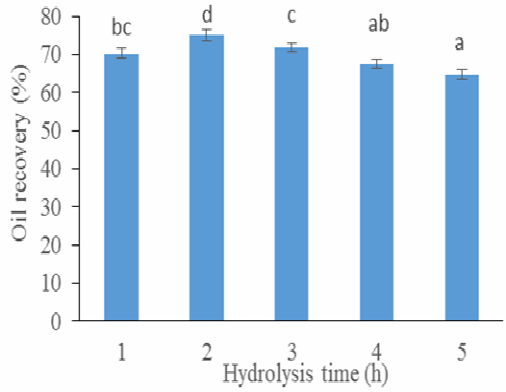

Figure 4. The influence of hydrolysis time on the oil recovery from barramundi by – product. Mean values with different superscript letter are significantly different (p<0.05)

The results indicated that there was a significant increase in oil recovery in the first 2 hours, followed by a decrease during the next 3 hours. The oil recovery from barramundi by - product reached the highest value (75.23%) after 2 hours of hydrolysis. However, when the hydrolysis time prolonged over 2 hours, the free oil recovery decreased significantly. After 5 hours of hydrolysis, the oil recovery only attained 64.65%. These results implied that the hydrolysis time of 2 hours was sufficient to release a large amount of free oil from barramundi by - product. Mbatia *et al.* (2010) also reported that the initial stage of hydrolysis could be sufficient to release the lipids. The long hydrolysis time did not improve the oil recovery but resulted in a colour change of hydrolysate solution to brow [7]. Šližyte *et al.* (2005) reported that the reduced release of lipids may be due to the formation of lipid - protein

complex [9]. The decrease in amount of free oil after 2 hours may be due to interaction of released oil with hydrolyzed proteins during hydrolysis. According to Dumay *et al.* (2009), it is not beneficial to perform a long hydrolysis to obtain the highest oil release. Indeed, the tissue disruption obtained at the beginning of the proteolysis appears sufficient to release the lipids [10]. Batista *et al.* (2009) reported that the lipid recovery was 35% after 1 hour of hydrolysis of sardine by - product with Protamex and only a small increase of 5% was observed in the next 3 hours [3]. The results in this study suggested that the optimal hydrolysis time for oil extraction from barramundi by - product was 2 hours.

In brief, the optimal hydrolysis conditions for oil extraction from barramundi by-product were the water/material ratio of 50%, enzyme/material ratio of 0.3%, hydrolysis temperature of 60^0C and hydrolysis time of 2 hours.

3.2. Chemical criteria of fish oil extracted from barramundi by - product

The chemical criteria of fish oil extracted from barramundi by - product with the optimal hydrolysis conditions is shown in table 1.

Table 1. Chemical criteria of fish oil extracted from barramundi by - product

Chemical criteria	Content
Free fatty acid (%)	1.25±0.12
Acid value (mg KOH/g)	2.49±0.24
Peroxide value (meq O_2/kg)	1.47±0.12
Iodine value (g I_2/100g)	121.85±1.87
Saponification value (mgKOH/g)	192.64±2.15

The free fatty acid content in oil is one of the most important quality parameters to value the quality of oil because the free fatty acid are more susceptible to oxidation than esterified fatty acids. The free fatty acid content should range between 1 and 7% but usually ranges between 2 and 5% [11]. The result in this study demonstrated that the amount of free fatty acid in the barramundi by - product oil was low (1.25%). The lower free fatty acid content showed higher quality and lower further oxidation.

The acid value is a measure of the hydrolysis that had occurred in the oil and is defined as the number of milligrams of potassium hydroxide required to neutralize the free fatty acids in 1 g of oil. The acid value of barramundi by-product oil was found to be 2.49 mg KOH/g, which was below the acceptable limit of 7 - 8 mg KOH/g reported by Bimbo and Crowther (1991) [12].

The peroxide value is commonly used to determine the rancidity of oil and is expressed in milli equivalent of active oxygen per kg of oil. The maximum limit of peroxide value of crude oil is 8 meq O_2/kg to be acceptable for human consumption [13]. The oil extracted from barramundi by-product had a peroxide value of 1.47 meq O_2/kg, which was still within the acceptable quality limit. This indicated that the fish oil extracted from barramundi by-product had low lipid oxidation rate.

The iodine value is a measure of degree of unsaturation of the oil and is defined as grams of iodine absorbed by 100 g of oil. Barramundi by - product oil had a iodine value of 121.85 g I_2/100 g, which was lower than that of mackerel oil (134 g I_2/100 g) [14].

Saponification is the process of breaking down a neutral oil into glycerol and fatty acids by alkali treatment. Saponification value represents the number of milligrams of potassium hydroxide required to saponify 1 g of oil. The oil extracted from the barramundi by-product had a saponification value of 192.64 mg KOH/g, which was higher than that of sardine oil (186.85 mg KOH/g) [6]. Saponification values of the hilsa fish oils from different parts were found to be arranged from 180.28 to 194 [15].

3.3. Fatty acid composition of fish oil extracted from barramundi by - product

Fatty acid composition of the oil recovered from barramundi by - product at optimum hydrolysis conditions is shown in table 2.

Table 2. Fatty acid composition of fish oil extracted from barramundi by - product

Fatty acids	Content (% total fatty acids)
C14:0 (myristic)	2.65±0.12
C16:0 (palmitic)	24.34±0.21
C18:0 (stearic)	8.05±0.14
C20:0 (arachidic)	0.50 ±0.08
C24:0 (linoceric)	1.24±0.13
C16:1 ω 7 (palmitoleic)	4.75±0.21
C18:1 ω 9 (oleic)	9.65±0.25
C18:1 ω 7 (vacenic)	3.38±0.13
C20:1 ω 9 (gadoleic)	1.56±0.06
C22:1 ω 9 (erucic)	2.57±0.10
C24:1 ω 9 (nervonic)	2.84±0.08
C18:2 ω 6 (linoleic)	2.94±0.16
C18:3 ω 3 (linolenic)	2.26±0.10
C20:4 ω 6 (arachidonic)	6.26±0.15
C20:5 ω 3 (eicosapentaenoic, EPA)	4.67±0.15
C22:4 ω 6 (docosatetraenoic)	2.34±0.11
C22:5 ω 3 (docosapentaenoic)	2.25±0.13
C22:6 ω 3 (docosahexaenoic, DHA)	17.75±0.23
Total saturated fatty acids	36.78±0.28
Total monounsaturated fatty acids	24.75±0.35
Total polyunsaturated fatty acids	38.47±0.33
Axit béo ω 3	26.93±0.35
Axit béo ω 6	11.54±0.17

The results indicated that the content of saturated fatty acids in the oil recovered from barramundi by - product was 36.78% of total fatty acids. Palmitic acid was the highest among the saturated fatty acids with the content of 24.34% followed by stearic acid with the content of 8.05%. The content of monounsaturated fatty acids was 24.75%. The most abundant monounsaturated fatty acid was oleic acid with the content of 9.65%. The content of polyunsaturated fatty acids was 38.47% of total fatty acids. Néchet *et al.* (2007) reported that the contents of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids in the oil from liver of ray (*Himantura bleekeri*) were 45.32%, 22.84% and 31.85% of total fatty acids, respectively [16]. The results in this study demonstrated that the fatty acids with high contents in barramundi by - product oil were palmitic acid, docosahexaenoic acid and oleic acid. The oil recovered from barramundi by - product had the omega-3 fatty acid content of 26.93% and omega-6 fatty acid content of 11.54%.

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) content of barramundi by - product oil were 17.75% and 4.67%, respectively. This showed that the barramundi by - product oil was a good source of DHA and EPA. DHA and EPA are known as essential fatty acids for human. DHA has an important role in the development of the brain and the nervous system. EPA plays an important role in the prevention of cardiovascular diseases. The contents of DHA and EPA in liver oil of ray (*Himantura bleekeri*) were found to be 15.62% and 4.10%, respectively [16]. Sun *et al* (2006) reported that the DHA and EPA contents in the oil extracted from salmon viscera were 6.99% and 7.91%, respectively [17]. Khoddami *et al.* (2012) showed that the DHA and EPA contents in the tuna head oil were 15.7% and 1.48%, respectively [18].

4. CONCLUSION

Barramundi by - product generated from fish processing industry could be utilized as a good source for recovery of fish oil. The oil recovery from barramundi by - product using Alcalase was significantly affected by the hydrolysis conditions including water to material ratio, enzyme to material ratio, hydrolysis temperature and hydrolysis time. The optimal parameters for oil extraction from barramundi by - product were the water/material ratio of 50%, enzyme/material ratio of 0.3%, hydrolysis temperature of 60^0C and hydrolysis time of 2 hours. Under these optimum

conditions, the fish oil extracted from barramundi by - product had a high content of omega-3 fatty acids (26.93%), especially DHA (17.75%). The oil from barramundi by-product could be used in aquaculture feed or used as an ingredient in food industries after it is refined.

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DISTRIBUTION OF PKS-I, PKS-II AND NRPS GENES IN 23 ACTINOBACTERIAL STRAINS SELECTED FROM SPONGES IN THE HA TIEN SEA, VIETNAM

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ABSTRACT

Marine actinobacteria are the most economically and biotechnologically valuable prokaryotes receiving much attention for their capacities of antibiotics and enzyme inhibitors. A total of 198 endophytic actinomycetes was isolated from 44 samples of 5 different sponge species in Ha Tien Sea, Vietnam. Twenty - three actinobacterial isolates against at least two of the five tested bacteria with moderate to high resistance were selected and characterized by the genetic sequencing method. They were identified to be belonged to 4 families: Actinomycetaceae, Microbacteriaceae, Nocardiaceae, and Gordoniaceae. The difference of present rate of genes including PKS-I, PKS-II and NRPS gene, was found as follows: 16/23 strains (with 69.6%), 14/23 strains (with 67.8%), 11/23 strains (with 47.8%) respectively, three strains had three PKS-I, PKS-II and NRPS genes, especially *Gordonia bronchialis* HD2.5a strain (Gordoniaceae) was the best strain in the resistance with 4/5 human diseases microbes. These new cultures can be employed as bioactive resources against pathogens, particularly in relation to food - borne diseases and human health.

Keywords: *Actinobacteria, antimicrobial activity, marine sponge, non - ribosomal peptide synthetase, polyketide synthetase.*

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1. INTRODUCTION

Most currently marketed antibiotics are natural products of microbial origin and >120 of the most important medicines in use today are obtained from terrestrial microorganisms [1 - 3]. The epidemiology of antibiotic - resistant bacteria and the need for confirmed test results [4]; leading to the global concern that we may soon be facing a post - antibiotic era with reduced capabilities to combat microbes. Therefore, a concerted worldwide search for new antibiotics from microbial origin is on - going, with focus on the potential of marine and terrestrial bacteria as sources for novel metabolites with interesting biological and pharmaceutical properties [5, 6]. Actinomycetes are a group of aerobic, branched, unicellular Gram - positive bacteria with high percentage of G+C (70%) in their genetic material. This bacterium has many important roles in various industries because of its ability to produce a few diverse metabolite compounds. These metabolite compounds have benefits such as antibiotics, antifungal, antiviral, anticancer, enzymes, immune suppressants and other compounds that are beneficial in industry [7]. It has become increasingly difficult to find actinobacteria with a great capacity to produce new antibiotics [8]. These actinobacteria from extreme environments were found to exhibit a unique source of novel biologically active compounds [8]. The rare actinobacteria could produce active metabolite, which has the property of diverse, unique, unprecedented, and occasionally complicated compounds usually with low toxicity. Sponges are constantly filtering bacteria from the water column and are home to a diversity of microbial symbionts [9]. The remarkable biotechnological potential of actinomycetes for drug discovery has been observed since the 1960s and since that time,

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actinomycetes have been responsible for more than 70% of all antibiotics discovered [2]. Marine actinobacteria are the most economically and biotechnologically valuable prokaryotes.

The method applied to study the distribution of PKS and NRPS biosynthetic systems was suggested in a collection of wild type actinomycetes isolated from tropical soil samples [10]. The antimicrobial activity and amplifying genes coding for PKS-I, PKS-II and NRPS showed that endophytic actinomycetes isolated from medicinal plants in Panxi plateau performed as valuable reservoirs of novel bioactive compounds [11].

This research was conducted to investigate the culturable diversity of actinobacteria from sponges of the Ha Tien Sea, Vietnam and to analyse genes coding for PKS-I, PKS-II and NRPS gene actinobacteria as well as to explore the potential use of these newly sponge associated actinobacteria as a novel source of bioactives.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 5 different sponge species were collected by scuba diving from the Ha Tien Sea at a depth from 0.5 m to 1.0 m under surface water, including 4 sites: Dam island (10°13′81N; 104°49′56’90”E), Heo island (10°17′89”N; 104°53′18’E), Nghe island (10°02’96” N; 104°55’62” E) and Nui Den (10°37′50” N; 104°44′58” E). The samples were placed into plastic bags and transported to the laboratory using an ice box and stored at -20°C until analysis.

2.2. Isolation of Actinobacteria

Starch Casein agar medium [12] was used for the isolation of sponge-associated actinobacteria. It was supplemented with Aginalxic (0.5 mg/L) and Nystatin (0.5 mg/L) to inhibit fungi and Gram - negative bacteria. Sponge samples were rinsed with sterile natural seawater to remove the microbes loosely attached to the surface. Subsequently, a few tissue cubes were excised from different sections (including cortex and endosome) of the sponge samples. They were cut into pieces and aseptically ground using sterilized pestles and mortars. Actinobacteria were isolated by means of serial dilution and plating techniques. The inoculated plates were incubated at 28°C for 3 - 6 weeks. The colonies bearing distinct morphological characteristics were picked up and transferred to freshly prepared media until pure cultures were obtained.

2.3. Screening assays for antibacterial activity

The bioactivity of bacterial isolates was examined [13, 14]. The pathogenic bacteria including *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Salmonella typhimurium* and *Candida albicans*, were provided by College of Aquaculture and Fisheries, Biotechnology Research and Development Institute (Can Tho University) and Can Tho Center for Technology, Standard, Quality (Department of Science and Technology, Can Tho city). The liquid cultures were grown with shaking at 150 rpm for 7 - 14 days depending on their growth rate at 30°C. The broth was centrifuged in 50 mL falcon tubes at 4,193 xg for 15 min at room temperature (28 - 32°C); Megafuge 1.0 R, Heraeus) and the supernatant was stored at 4°C. The bacterial and fungal test organisms were plated in Mueller Hinton agar medium and Potato Dextrose agar medium, respectively. Antimicrobial extract was added to the wells, then the plates were incubated at 4°C for 2 hours for diffusion of antimicrobial extract and observed for the zones of inhibition at 28°C for 48 hours.

2.4. The agar well diffusion method

The active isolates were cultured by the method given in the previous step. The supernatants were used for testing extracellular antimicrobial activity by the agar well diffusion method. By using a sterile cork borer, wells were punctured in appropriate agar medium previously seeded with one of the test organisms. One hundred microliters of the culture supernatants were added to each well. The plates were then incubated at 4°C for at least 2 hours to allow the diffusion of crude extracts followed by incubation for 24 hours at 37°C for bacteria and 48 hours at 28°C for yeast. The diameters of inhibition zones were monitored and measured [15].

2.5. Genomic DNA Extraction

To prepare cultures for the extraction of genomic DNA from the isolates, a single colony was transferred to a 5 mL microtube with 1 mL of liquid medium from which the isolate was originally picked up. The cultures were incubated for 3~5 days at 28°C with shaking at 180 rpm. Bacterial cells from these cultures were collected by centrifugation and genomic DNA was extracted [16].

2.6. 16S rRNA Gene Amplification and Sequencing

Amplification of 16S rDNA by PCR was carried out using the universal primers 27F [17] and 1492R [18], implementation consists of the following components and processes: The 50 µL reaction mixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of dNTP, 500 nM of each primer (Fermentas) and 20 ng DNA. The following programs: 5 min at 95°C and 30 cycles of denaturizing for 30 s at 95°C, annealing for 2 min at 55°C, extension for 90 s at 72°C and a final extension for 10 min at 72°C in C1000 Thermal Cycler (Bio-Rad). Aliquots (10 µL) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures.

The following actinobacteria - specific primers were used for the amplification of actinobacterial 16S rRNA gene fragment [19]. Cycling conditions were as follows: Initial denaturation for 4 min at 95°C for and 30 cycles of denaturizing of 45 s at 95°C, annealing of 45 s at 68°C, 1 min at 72°C and a final extension of 8.5 min at 72°C.

S-C-Act-0235-a-S-20(5′-CGCGGCCTATCAGCT TGTTG 3′) and

S-C-Act-0878-a-A-19(5′-CCGTACTCCCCAGGC GGGG-3′)

2.7. Sequence analysis

The 16S rRNA gene sequences were compared with those from the type strains available in NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) [20]. For the phylogenetic analysis, multiple sequence alignment was performed using CLUSTALX, version 1.81. Phylogenetic trees were constructed using Mega 7.0 [21]. The consistency of the trees was verified by bootstrapping (1000 replicates) for maximum likelihood [10, 11].

2.8. Statistical analysis

The experimental results were analyzed as ANOVA with the isolates and with levels of diameters of inhibition zones. All analyses were conducted using the programme MSTATC, Minitab 16. The data were considered significantly different at $p < 0.01$. Duncan test at $p = 0.01$ was used to differentiate between statistically.

3. RESULTS AND DISCUSSION

A total of 198 isolates of actinomycetes were purified from 44 sponge samples collected at 4 sites. The numbers of samples and isolates (samples/isolates) were obtained at Dam island, Nghe island, Heo island and Nui Den as follow: 15/72, 11/33, 10/19 and 8/18, respectively.

Table 1. Antimicrobial activity of 23 sponge-derived actinomycete isolates

No	Ampersand Isolates	Inhibition zone diameter [D = d_1 - d_2] (mm)				
		Bacillus cereus	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
1	ND1.1a	15.0 ^{f}	-	14.0 ^{a}	-	23.0
2	ND1.3b	14.0 ^{g}	-	-	14.0 ^{d}	16.0 ^{h}
3	ND1.5a	17.0 ^{d}	-	-	11.0 ^{g}	12.0 ^{k}
4	ND1.7a	21.0 ^{a}	-	9.0 ^{d}	12.0 ^{f}	23.7 ^{b}

No	Ampersand Isolates	Inhibition zone diameter [D = d_1 - d_2] (mm)				
		Bacillus cereus	*Staphylococcus aureus*	*Escherichia coli*	*Salmonella typhimurium*	*Candida albicans*
5	ND1.7b	13.0 [h]	-	14.0 [a]	-	25.7 [a]
6	ND2.6c	17.0 [d]	-	9.0 [d]	14.0 [d]	21,7 [d]
7	ND2.7c	21.0 [a]	-	12.0 [b]	12.0 [f]	22.0 [d]
8	HD1.2c	9.0 [i]	-	7.0 [f]	10.0 [h]	26.0 [a]
9	HD1.3d	15.0 [f]	-	8.0 [e]	19.0 [b]	14.0 [j]
1	HD1.3e	20.0 [b]	-	11.0 [c]	15.0 [c]	19.0 [e]
11	HD1.5c	17.0 [d]	-	9.0 [d]	22.0 [a]	-
12	HD1.6a	21.0 [a]	-	8.0 [e]	4.0 [l]	18.0 [f]
13	HD2.1a	14.0 [g]	-	5.0 [h]	19.0 [b]	18.0 [f]
14	HD2.3a	13,0 [h]	-	14.0 [a]	6.0 [k]	16.0 [h]
15	HD2.3b	20.0 [b]	-	6.0 [g]	15.0 [c]	-
16	HD2.3c	18.0 [c]	-	6.0 [g]	9.0 [i]	26.0 [a]
17	HD2.4a	17.0 [d]	-	7.0 [f]	9.0 [i]	17.0 [g]
18	HD2.5a	15.0 [f]	-	12.0 [b]	14.0 [d]	14.0 [j]
19	HD2.5d	15.0 [f]	-	-	14.0 [d]	12.0 [k]
2	HD2.7d	16.0 [e]	-	4.0 [i]	13.0 [e]	15.0 [i]
21	HD2.8p	14.0 [g]	-	11.0 [c]	10.0 [h]	14.0 [j]
22	HD2.9a	7.0 [k]	-	-	12.0 [f]	14.0 [j]
23	N10b	7.0 [k]	12.0 [a]	-	-	-
24	Control	8.0 [j] Ampicillin	12.0 [a] Ampicillin	7.0 [f] Tetracycline	8.0 [j] Tetracycline	10.0 [l] Fluconazole
	CV%	2.05	5.89	2.45	3.74	1.43

Note: Sample collection site letters, isolation sample numbers, and isolate species number digits. Means within a column followed by the same letter/s are not significantly different at 99%; Standard deviations must be presented together with means 1.43~5.89. D = diameter of inhibition zone of isolates, d1 = diameter of inhibition zone, d2 = diameter of well, ND: not detected.

Almost all their colonies have round shapes; milky, white clear and yellow, entire, or lobate margin; diameter size of these colonies varied from 0.2 to 3.0 mm and all of them have Gram - positive. Twenty - three of 198 tested isolates could produce antimicrobial active metabolites inhibiting at least two of the test pathogens. Over fifty percent isolates could inhibit the growth of Gram - positives, 20/198 isolates were actively against *Candida albicans,* and 21 isolates showed activity against two among five following pathogens including *Bacillus cereus, Staphylococcus aureus, Escherichia coli, Salmonella typhimurium* and *Candida albicans* (Table 1).

Based on the result from table 1, 23 isolates were chosen to analyze for PCR technique and sequencing. The result showed that 23 selected isolates belonged to the families Actinomycetaceae 65.2% (15 strains), Microoccupied 8.7% (2 strains), Nocardiaceae 21.7% (5 strains), Gorgoniaceae 4.4% (1 strain), respectively (Figure 1).

Family Actinomycetaceae, genus *Streptomyces* with 15 strains (65.2%) was the most prominent genus, this result is suitable with the [22] result' (occupied over 95% species in class Actinomycetales) and family Gordoniaceae had the lowest ratio (4.4%) (1 strain).

A total of 23 isolates were chosen for identification due to their antimicrobial activity. The fragments of 600 - 620 bp 16S rRNA were obtained from PCR, antimicrobial activity and presence of biosynthetic gene sequences, the detection levels of PKS-I and PKS-II and NRPS sequences by specific amplification of total DNA: Type I (PKS-I), polyketide synthase type II (PKS-II) and nonribosomal peptide synthetase (NRPS) with PCR (Figure 2, 3, 4).

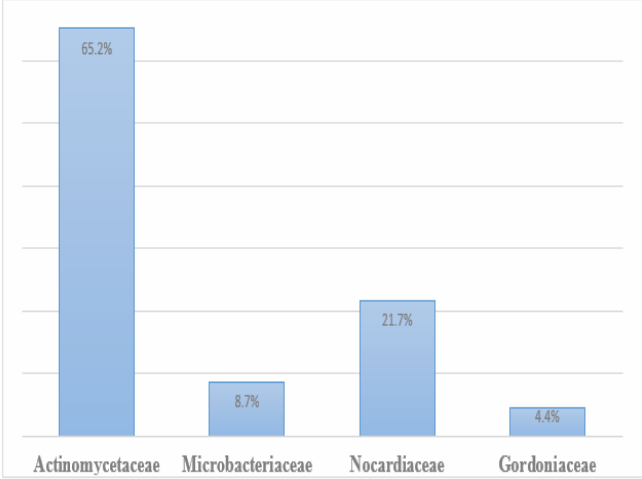
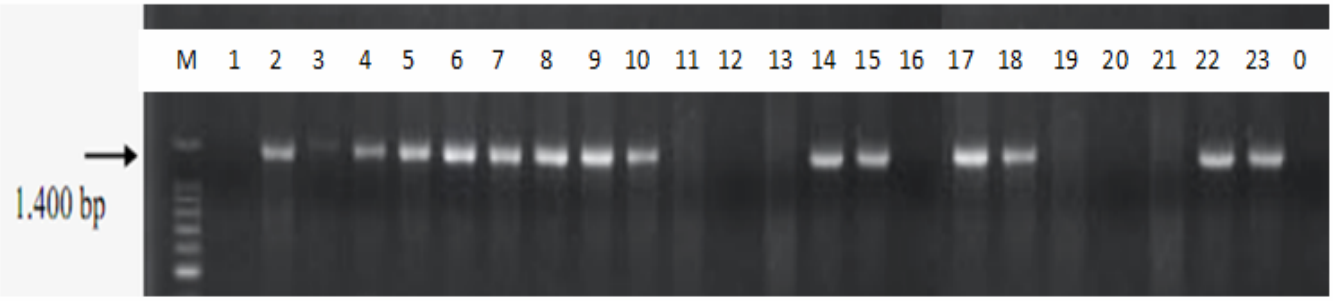


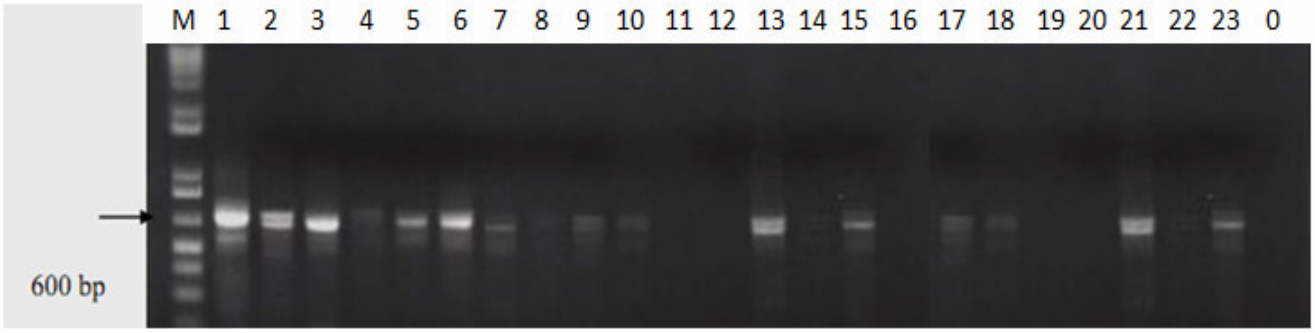
Figure 1. Ratio of the Actinomycete strains were selected to sequence

The research findings on the diversified collection of sponge-associated actinobacteria isolated from the Ha Tien Sea, Kien Giang province of Vietnam indicate the important sources for searching such potential resources of biological active compounds.



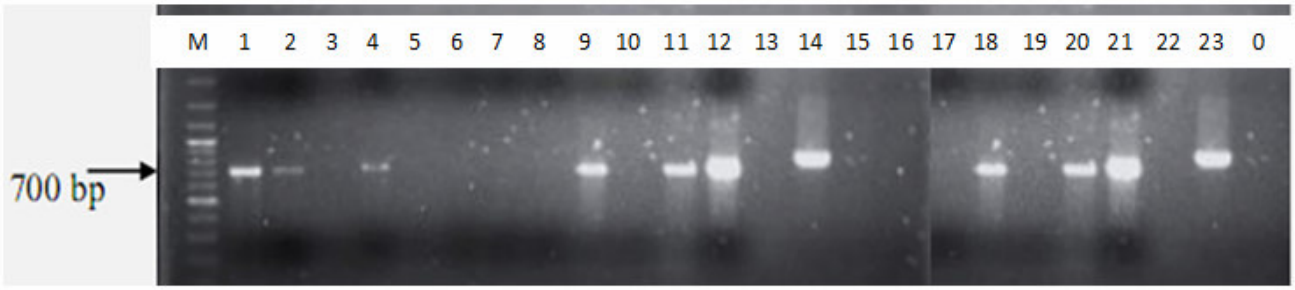
(M: Ladder 100 bp, 1: N10b, 2: HD2.9a, 3: HD2.8p, 4: HD2.7d, 5: HD2.5d, 6: HD2.5a, 7: HD2.4a, 8: HD2.3c, 9: HD2.3b,10: HD2.3a, 11: HD2.1a, 12: HD1.6a, 13: HD1.5c, 14: HD1.3e, 15: HD1.3d, 16: HD1.2c, 17: ND2.7c, 18: ND2.6c, 19: ND1.7b, 20: ND1.7a, 21: ND1.5a, 22: ND1.3b, 23: ND1.1a, 0: Negative Control)

Figure 2. Agarose gel electrophoresis of PCR products from DNA isolated from representative actinomycete strains: Selective amplification of the 1200 - 1400 bp fragments using primers for NKS-I gene of 23 selected actinomycete strains



(M: Ladder 100 bp plus, 1: HD2.1a, 2: HD2.3a, 3: HD2.3b, 4: HD2.3c, 5: HD2.4a, 6: HD2.5a, 7: HD2.5d, 8: HD2.7d, 9: HD2.8p,10: HD2.9a, 11: ND2.7c, 12: ND2.6c, 13: ND1.7b, 14: ND1.7a, 15: ND1.5a, 16: ND1.3b, 17: ND1.1a, 18: HD2.1c, 19: HD1.3d, 20: HD1.3e, 21: HD1.5c, 22: HD1.6a, 23: N10b, 0: Negative Control).

Figure 3. Agarose gel electrophoresis of PCR products from DNA isolated from representative actinomycete strains: Selective amplification of the 600 bp fragments using primers for NKS-II gene of 23 selected actinomycete strains



(M: Ladder 100 bp plus, 1: ND2.7c, 2: ND2.6c, 3: ND1.7b, 4: ND1.7a, 5: ND1.5a, 6: ND1.3b, 7: ND1.1a, 8: HD1.2c, 9: HD1.3d,10: HD1.3e, 11: HD1.5c, 12: HD1.6a, 13: HD2.1a, 14: HD2.3a, 15: HD2.3b, 16: HD2.3c, 17: HD2.4a, 18: HD2.5a, 19: HD2.5d, 20: HD2.7d, 21: HD2.8p, 22: HD2.9a, 23: N10b, 0: Nagative control)

Figure 4. Agarose gel electrophoresis of PCR products from DNA isolated from representative actinomycete strains: Selective amplification of 700 - 800 bp fragments using primers for NRPS adenylation sequences of 23 selected actinomycete strains

Homology searches of 16S rRNA gene sequence of selected in GenBank by BLAST (Table 2) revealved that they had similarity to sequences of genus *Streptomyces* (15 strains) and 3 families including Microbacteriaceae (*Microbacterium tumbae*) (2 strains), Nocardiaceae (*Rhodococcus*) (5 strains) and Gordoniaceae (*Gordonia bronchialis*) (1 strain).

According to [23], the diversity of actinobacteria in *Xestospongia muta* and *Xestospongia testudinaria* were very high. The 106 actinobacteria isolates were isolated from 7 genera of *Hymeniacidon perleve* sponge [24]. Identified 181 species of actinomycete with 3 genera from 5 species of sponge in China [25]. Analysis of the diversity of microbes from 2 strains of Red sea sponges (*Hyrtios erectus* and *Amphimedon* sp.), showed that 35 species of actinomycete in 4 genera. In our study, 198 actinomycete isolates isolated from sponges in Ha Tien Sea, 23 isolates were identified having high activity against human disease microbes.

Table 2. Phylogenetic affiliation of 23 isolates on the basis of 16S rRNA gene sequences by using BLAST programme in the GenBank database based on sequences similarity

No	Taxonomic group and strain	Closest species relative	Nucleotide number (bp)	Similarity (%)
	Actinomycetaceae			
1	ND1.1a	*Streptomyces coelicolor* ND1.1a	606	100
2	ND1.3b	*Streptomyces griseoaurantiacus* ND1.3b	604	100
3	ND1.5a	*Streptomyces ramulosus* ND1.5a	608	100
4	ND1.7a	*Streptomyces tateyamensis* ND1.7a	602	100
5	ND1.7b	*Streptomyces ambofaciens* ND1.7b	603	100
6	HD1.2c	*Streptomyces recifensis* HD1.2c	605	100
7	HD1.3d	*Streptomyces olivaceus* HD1.3d	605	100
8	HD1.3e	*Streptomyces althioticus* HD1.3e	603	100
9	HD1.5c	*Streptomyces tateyamensis* HD1.5c	604	99.83
10	HD1.6a	*Streptomyces flaveolus* HD1.6a	604	100
11	HD2.1a	*Streptomyces chumphonensis* HD2.1a	609	100
12	HD2.3b	*Streptomyces olivaceus* strain HD2.3b	602	100
13	HD2.3c	*Streptomyces coelicolor* HD2.3c	606	100
14	HD2.9a	*Streptomyces qinglanensis* HD2.9a	604	100
15	N10b	*Streptomyces variabilis* N10b	609	100
	Microbacteriaceae			
16	ND2.7c	*Microbacterium tumbae* ND2.7c	618	100
17	HD2.8p	*Microbacterium tumbae* HD2.8p	610	100
	Nocardiaceae			
18	ND2.6c	*Rhodococcus hoagii* ND2.6c	606	100

No	Taxonomic group and strain	Closest species relative	Nucleotide number (bp)	Similarity (%)
19	HD2.3a	*Rhodococcus hoagii* HD2.3a	604	100
20	HD2.4a	*Rhodococcus rhodochrous* HD2.4a	603	100
21	HD2.5d	*Rhodococcus pyridinivorans* HD2.5d	604	100
22	HD2.7d	*Rhodococcus hoagii* HD2.7d	613	100
	Gordoniaceae			
23	HD2.5a	*Gordonia bronchialis* HD2.5a	604	100

The result presenting at figure 5 showed that 23 actinobacterial strains described two clusters: Cluster A had 14 strains, and cluster A divided two small clusters: Cluster A1 had 12 strains including 10 *Streptomyces* strains, *Microbacterium tumbae* HD2.8p strain and *Rhodococcus rhodochrous* HD2.4a strain. Cluster A2 had two strains: *Rhodococcus hoagii* HD2.7d strain and *Rhodococcus hoagii* ND2.6c strain. Cluster B had 9 strains and this cluster divided two small clusters: Cluster B1 had 4 strains with *Microbacterium tumbae* ND2.7c strain located in the separate clusterand three *Streptomyces coelicolor* HD2.3c (originated from Dam island), *Streptomyces coelicolor* ND1.1a strain (from Den mountain) and *Streptomyces variabilis* N10b strain (from Nghe island), this result showed that three strains had other originsfar from many kilometers, but they had genetic relationship nearly. Cluster B2 had 5 strains including *Rhodococcus rhodochrous* HD2.4a strain in separate cluster, two strains *Streptomyces recifensis* HD1.2c strain and *Streptomyces olivaceus* HD1.3d strain (in smaller cluster of cluster B2) and two strains: *Rhodococcus hoagii* HD2.3a strain and *Rhodococcus pyridinivorans* HD2.5d strain in smaller cluster in cluster B2, however all of five strains in cluster B2 had original site (Dam island), this result showed that they had close genetic relationship.

These actinobacteria strainshad genetic relationship closely even though they isolated from the sites far from Dam island to Nui Den, however many strains isolated at one site, but they were located in two separate clusters in the phylogenetic tree. These changes were based on the analyses of sequences of actinobacteria strainswith diversity of sponge - associated actinobacteria and seawater condition [26].

Streptomyces sp. isolated from sponges at Egypt sea, which had high resistance ability to *Staphylococcus aureus* and *Candida albicans*, they also against to *Enterococcus faecalis*, *Leishmania major*, *Trypanosoma brucei* [27]. Besides, *Streptomyces* sp. isolated from *Halichondria panicea*sponge having antimicrobial activity [28, 29], *Streptomyces* sp. Againstto *Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis.* In our results showed that *Streptomyces* sp. had high resistance activity to *Bacillus cereus* and *Salmonella typhimurium*. In addition, *Brevibacterium* sp. isolating from *Callyspongia*sponge identified to against *Escherichia coli*and *Staphylococcus aureus*from 2 compounds: 6-hydroxymethyl-1-phenazine-carboxamide and 1,6-phenazinedimethanol [30]. *Brevibacterium* sp. also isolated *Neopetrosia exigua*sponge in Mauritius sea, East Africa [31]. *Microbacterium* sp. isolated *Erylus discophorus*sponge [32] and *Microbacterium* sp. also isolated *Spongia* sp. sponge at Portugal sea [33], *Microbacterium* sp. identified to against to *Staphylococcus aureus* [26].

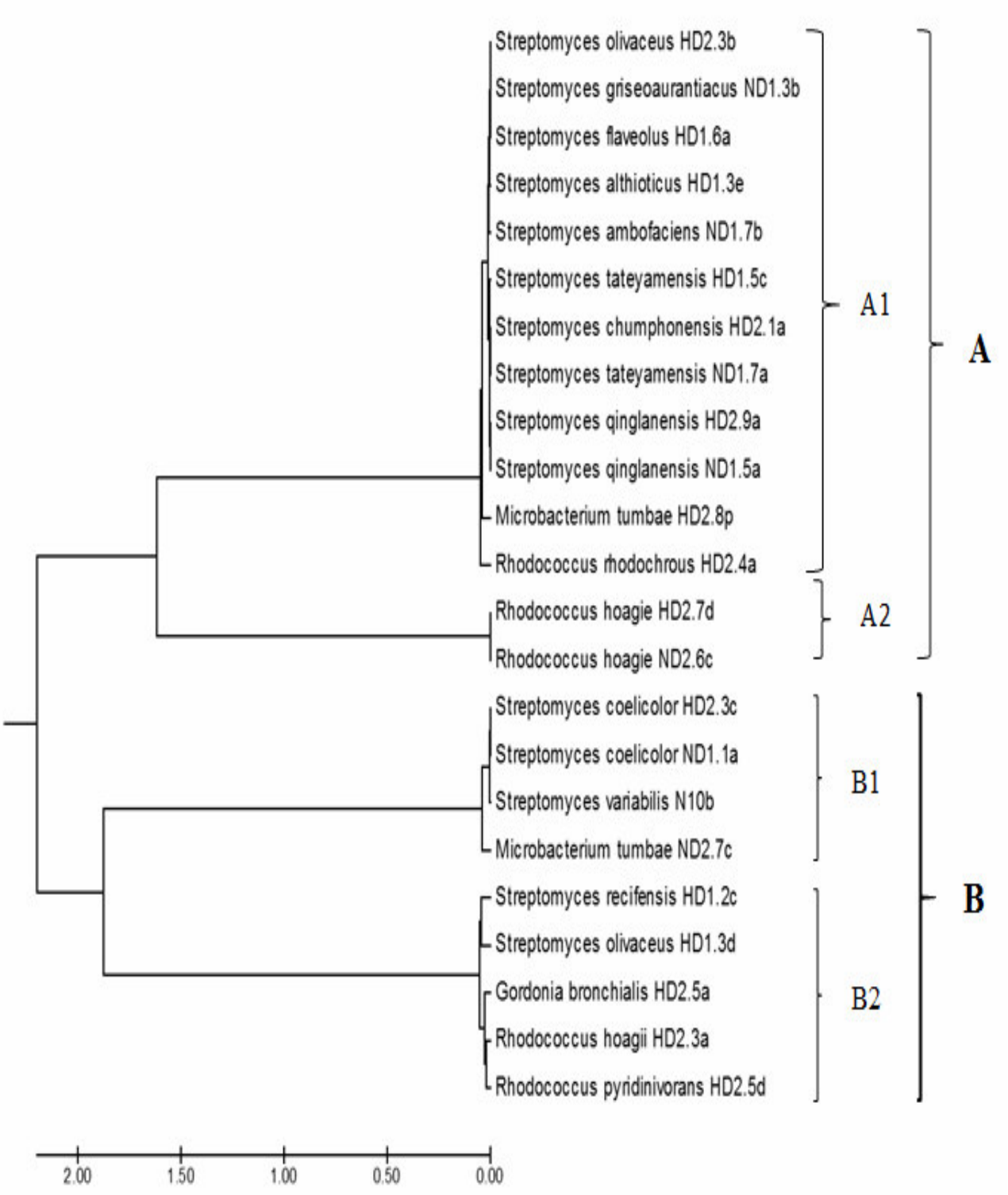


Figure 5. The maximum likelihood phylogenetic tree of partial 16S rRNA gene sequences of 23 actinobacteria isolates with primers (SC-Act-0235-aS-20 and SC-Act-0878-aA-19) isolated from sponges of the Ha Tien Sea and closely related type strains. Numbers in the figure refer to percentage bootstrap values in the MEGA 11 program which were calculated for 1000 replicates and nucleotide spacing

The presence of PKS-I, PKS-II and NRPS genes in all of *Streptomyces,* Microbacteriaceae, Nocardiaceae and Gordoniaceae (Figure 6 and table 3).

Table 3. The presence of PKS-I, PKS-II and NRPS genes of 23 selected actinomycete strains

No	Taxa	Gen PKS-I K1F/M6R (1.200 - 1.400 bp)	Gen PKS-II KSαF/KSαR (600 bp)	Gen NRPS A3F/A7R (700 - 800 bp)
	Actinomycetaceae			
1	*Streptomyces coelicolor* ND1.1a	+	+	-
2	*Streptomyces griseoaurantiacus* ND1.3b	+	-	-
3	*Streptomyces ramulosus* ND1.5a	-	+	-
4	*Streptomyces tateyamensis* ND1.7a	-	+	+
5	*Streptomyces ambofaciens* ND1.7b	-	+	-
6	*Streptomyces recifensis* HD1.2c	-	+	-
7	*Streptomyces olivaceus* HD1.3d	+	-	+
8	*Streptomyces althioticus* HD1.3e	+	-	-
9	*Streptomyces tateyamensis* HD1.5c	-	+	+
10	*Streptomyces flaveolus* HD1.6a	-	-	+
11	*Streptomyces chumphonensis* HD2.1a	-	+	-
12	*Streptomyces olivaceus* strain HD2.3b	+	+	-
13	*Streptomyces coelicolor* HD2.3c	+	-	-
14	*Streptomyces qinglanensis* HD2.9a	+	+	-
15	*Streptomyces variabilis* N10b	+	+	+
	Microbacteriaceae			
16	*Microbacterium tumbae* ND2.7c	+	-	+
17	*Microbacterium tumbae* HD2.8p	+	-	+
	Nocardiaceae			
18	*Rhodococcus hoagii* ND2.6c	+	-	+
19	*Rhodococcus hoagii* HD2.7d	+	-	+
20	*Rhodococcus hoagii* HD2.3a	+	+	+
21	*Rhodococcus rhodochrous* HD2.4a	+	+	-
22	*Rhodococcus pyridinivorans* HD2.5d	+	+	-
	Gordoniaceae			
23	*Gordonia bronchialis* HD2.5a	+	+	+

Ghi chú: "+": detected; "-": no detected

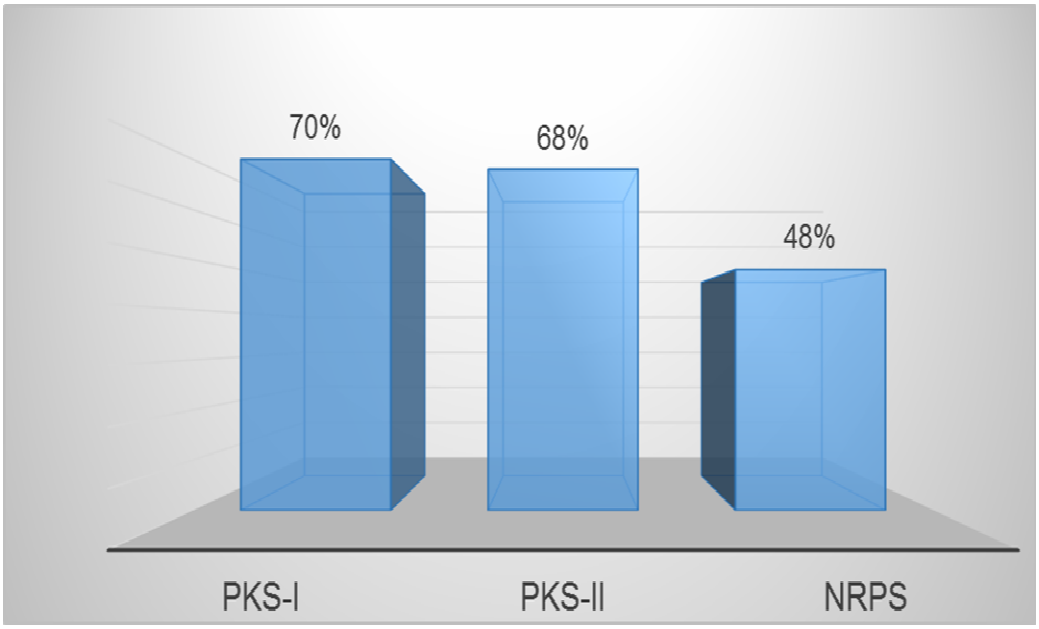


Figure 6. Percent of quantity strains presented in three kinds of primers for detection of PKS-I, PKS-II and NRPS gene

Using primers detected PKS-I gene on 8 strains of *Streptomyces* and all strains of 3 families Microbacteriaceae, Nocardiaceae and Gordoniaceae, with 16/23 strains having the presence of PKS-I gene (69.6%). The presence of PKS-II gene detected on 14/23 strains with KSαF/KSαR primers (60.9%), including 10 species of *Streptomyces*, 3 species of *Rhodococcus* and 1 strain of *Gordonia* (*Gordonia bronchialis* HD2.5a). 11/23 strains were detected when using primers with NRPS gene (47.8%), including 5 species of *Streptomyces*, 2 strains of *Microbacterium*, 3 strains of *Rhodococcus* and *Gordonia bronchialis* HD2.5a.

The result presenting at figure 6 showed that the highest PKS-I gene (occupied 70%) and the lowest NRPS gene (48%). This result also was similar to that of the previous studies about PKS-I, PKS-II and NRPS gene as [34], isolated and select the 41 strains of *Streptomyces* from herbal tree in tropical forest in Van Nam province, China having the ability of resistance to antimicrobial, tumor; among 31.7% strains made cell toxicity to against A549 cell; 29.3% to HL-60 cell, 85.4% to BEL-7404 cell, 90.2% to P388D1 cell and 65.9% strains resisted to *Escherichia coli*, 24.4% to *Staphylococcus aureus*, 31.7% to *Staphylococcus epidermidis*, 12.2% to *Candida albicans* but no strain resisted with *Klebsiella pneumoniae*. When testing the presence of PKS-I, PKS-II and NRPS gene with primers as K1F/M6R, KSαF/KSαRand A3F/A7R, the result showed that detection ratio were PKS-I (34.1%), PKS-II (63.4%) and NRPS (61.0%).

Using primer PKS-I and primer PKS-II to detect the presence of and NPRS gene of 210 actinomycete isolates from 33 other genera [10]. NRPS gene detected 167/210 isolates (79.5%) while 119/210 isolates were detected by PKS-I gene (56.7%). Almost all the species of *Streptomyces* were detected by NRPS and PKS-I gene (97% and 79%). While NRPS and PKS-I gene detected with 91% and 59% actinomycete isolates in Nocardiaceae.

In Viet Nam, studied *Streptomyces cavourensis* YBQ75 strain isolating from C*innamomum cassia* Presl in Yen Bai province, the result showed that this strain resisted to 5 human diseases bacteria as *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Enterobacter aerogenes, Proteus vulgaris* and the presence of PKS-I, PKS-II and NRPS gene in antibiotic synthesis, *Streptomyces cavourensis* YBQ75 had three of PKS-I, PKS-II and NRPS gene [35]. Our result also showed that three strains *Streptomyces variabilis* N10b, *Rhodococcus*

hoagii HD2.3a and *Gordonia bronchialis* HD2.5a had the presence of three PKS-I, PKS-II and NRPS gene. *Streptomyces variabilis* N10b strain against the *Staphycolococcus aureus* (while 22 strains did not resist with this human disease bacteria), *Rhodococcus hoagii* HD2.3a strain against the human disease microbes as *Bacillus aureus, Esccherichia coli* and *Candida albicans* while *Gordonia bronchialis* HD2.5a strain had high antimicrobial activity with 4 human diseases microbes (except *Staphycolococcus aureus*) as *Bacillus aureus, Esccherichia coli, Salmonell typhymurium* and *Candida albicans*, the results showed that *Gordonia bronchialis* HD2.5a strain (Gordoniaceae) was the best strain in the resistance with 4/5 human diseases microbes while *Streptomyces variabilis* N10b strain only against *Staphycolococcus aureus* and these strains need to be studied the bioactives which were extracted from them as new antibiotics.

4. CONCLUSION

Twenty - three actinomycete strains were selected with high antimicrobial activity, they had at least one in three PKS-I, PKS-II and NRPS gene, three strains had three PKS-I, PKS-II and NRPS gene, especially *Gordonia bronchialis* HD2.5a strain (Gordoniaceae) was the best strain in the resistance with 4/5 human diseases microbes and theyhad high potential for research of bioactive production.

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IMPLEMENTATION RESULTS OF THE NATIONAL COFFEE PRODUCT DEVELOPMENT PROJECT UP TO THE YEAR 2020 AND ORIENTATION OF PRODUCT RESEARCH AND DEVELOPMENT TO 2030

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ABSTRACT

All tasks of the national coffee product development project up to the year 2020 were implemented, except for one coffee variety that was not accredited due to requirements of the Law on Cultivation. As results, 14 technical protocols were accepted, including two technological advances, three ministerial-level ones. All protocols proved to be outstandingly effective more than 10% in production comparing to the old ones. For Robusta breeding activities, two clones *Xanh lun* and *Day* have been accredited by the Ministry of Agriculture and Rural Development for special circulation in the provinces of Dak Lak, Dak Nong, and Lam Dong. Regarding Arabica coffee, the TN9 variety has been tested for distinctness, uniformity, and stability (DUS) in Lam Dong, Kon Tum, Quang Tri and Son La provinces. Within the framework of the project, the total number of seedlings and seeds were provided to farmers and coffee growing organization in the Central Highlands region to grow/replant coffee with an estimated area of 13.000 - 15.000 hectares.

Keywords: *High quality coffee, national product, Vietnam.*

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1. INTRODUCTION

Recently, Vietnam's coffee industry has made remarkable progress in terms of yield, total area and productivity. By 2022, the country's coffee area is about 710 thousand hectares, with an average yield of more than 2.8 tons/ha, among the highest in the world [1]. Vietnam still accounts for about 14% of the global coffee market share, ranks second in green coffee export (after Brazil) and is the world's largest robusta coffee producer. In 2022, Vietnam exports about 1.8 million tons of commercial coffee to over 80 countries and territories around the world, with a turnover of about 4 billion USD. Coffee has become one of the country's important agricultural products, a key export product, creating jobs for millions of people [1].

Although highly ranked in the world in coffee production and export, the value that coffee brings back is not high. The coffee production system still has potentially unsustainable factors due to wasteful and inappropriate use of resources and inputs. Although there have been many studies on the current status of applying farming practices in coffee production, thereby providing the most reasonable solutions to improve coffee yield and quality resulting in increasing economic efficiency. However, the level of application is still limited and not synchronous. Producers are still limited in applying new advances and still practice farming based on experience, lacking of scientific basis. Besides, Vietnam's coffee quality is still low and non-uniform, especially the cup quality. There are many factors leading to this low quality such as not good varieties, nutrition, old aged plantation, diseases, harvesting, processing, etc. In addition, with the shifting economic structure towards industrialization and modernization, manual labor

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lacking will be an issue that will affect coffee production in Vietnam in the near future [2].

To solve this problem, the most important solutions to ensure efficient coffee production in Vietnam are to use new coffee varieties with high productivity and quality according to world standards. In addition, it is necessary to synchronously apply advanced farming techniques such as fertilization based on soil fertility, water saving irrigation, fertigation, and applying mechanization oriented techniques in production. At the same time, the establishment of wind-break trees, shade trees or intercropping to diversify harvested products in coffee plantations such as growing fruit trees with high economic value like durian, avocado... with appropriate density to increase income per unit area. This is also considered as a coffee farming solution to adapt to climate change, ensuring sustainable development of the coffee industry. In addition, pre-harvest measures, concentrated on uniform ripening treatment, and minimizing post-harvest losses of coffee have not been focused and fully researched. Therefore, application of these studies must synchronize the harvesting and processing process to ensure the effectiveness of technical measures [2].

In short, in order for Vietnam's coffee industry to develop more sustainably and increase added value for domestic consumption and export, the implementation of the science and technology project: "Technology for selecting and producing high-quality coffee and advanced coffee cultivation techniques with high productivity and quality" under the national product development program "Vietnamese high quality coffee" are essential. The project has been actively implemented for 3 years 2018-2020 and has had important results, contributing to sustainable coffee development in Vietnam.

2. OBJECTIVES OF NATIONAL COFFEE PRODUCTS PROGRAM

- Breeding and development of Robusta coffee varieties met with high quality criteria, grown in suitable ecological areas, and cultivated according to sustainable coffee production protocols, resistant to most important pests and diseases, adapted to climate change, accredited and protected as key cultivars in production.

- Breeding of Arabica coffee varieties met with high quality criteria (caffeine, physic, aroma), resistant to major pests and diseases, cultivated according to sustainable coffee production protocols, accredited and protected is main cultivars in the coming time.

- Development of synchronous packages of coffee production techniques covered from seedling use to harvesting with orientation of mechanization and GAP/BAP application to improve economic efficiency, sustainable production, and high-quality product production for domestic consumption and export.

- Development of advanced seedling and seed production protocols, creating high quality, disease-free coffee seedlings to provide for new planting and re-cultivation of coffee.

- Building of production models, demonstration models applying advanced technical protocols from seedling to cultivation and harvesting in order to transfer new technologies to production.

3. MAIN ACCOMPLISHMENTS

3.1. Research on breeding of high-quality Robusta coffee varieties for concentrated coffee growing regions in the Central Highlands

3.1.1. Selection, breeding and evaluation of starting materials

- Six high-quality Robusta clones have been selected from hybrid progenies including: L2H24C10, L2H36C1, L3H20C7, L4H6C10, L4H15C8 and L4H18C6. These clones have good growth characteristics, the average yield is 2.6 kg beans/tree. In the harvest season, the average cherries/dry beans ratio was 4.5, the weight of 100 beans was 20.4 g, the average bean rate on screen 16 was 89.5%, and especially the cup quality was, in accordance with the standard SCA scores, from 80.0 to 82.5 points, these clones have not shown leaf rust disease in the field [3].

- Nine drought tolerance Robusta clones have been selected from hybrid progenies: CH18C5,

CH18C16, CH20C10, CH20C22, CH38C20, CH41C17, CH41C26, C H43C4, CH43C8. These clones were completely wilted after 41 - 46 days of watering, clearly longer from the control variety with 31 - 33 days. The average yield was 1.73 kg of green beans/tree. In the harvest season, the average cherries/dry beans ratio was 4.5, the weight of 100 beans was 19.0 - 22.4 g, and the average bean rate on screen 16 was 90.2%. No leaf rust disease was observed in the field [3].

3.1.2. Selection of new coffee varieties

a) Prospective clones (from variety evaluation and comparison experiments):

Eight promising clones have been selected including: Lam H17C19, Quy H27C10, Duc H10C4, Long H8C2, TTGL H4C8 and Hoi H6C23, L4H15c2, L1H36C9. These clones have shown SCA standards score of cup quality >80 points, the average yield of the established crop is >3.0 tons of beans/ha, the weight of 100 beans of all clones is over 20.0 g, the rate of beans on screen 16 is >85%.

b) New Robusta coffee cultivars

Two Robusta cultivars of *Xanh lun* and *Day* have been selected with wide adaptability in the trial areas. The yield was >3.8 tons of green beans/ha in the first established season. the average yield shown in production was >4.5 tons of green beans/ha. The above 2 clones have SCA standards scores of cup quality from 80.0 to 82.5 points. The weight of 100 beans is from 22.5 to 27.8 g, the rate of beans on screen 16 >95%. These two clones *Xanh lun* and *Day* have been accredited exceptionally by the Ministry of Agriculture and Rural development for the provinces of Dak Lak, Dak Nong and Lam Dong according to Decisions No. 74/QD-TT-CCN and Decision No. 75/QD-TT-CCN dated April 20. 2021 [3].

3.1.3. New breed models

Thirty hectares of new Robusta coffee varieties have been established in 5 provinces of the Central Highlands. These new varieties can grow and adapt widely in the growing areas with an average yield of 3.20 tons of green beans/ha in the first harvest season, while the control clone TR11 reached only 2.48 tons of green beans/ha [3].

3.2. Research on breeding of high-quality Arabica coffee for production in the main growing regions

3.2.1. Evaluation, creation of starting materials and varieties comparison

- Five new arabica coffee materials originated from Lam Dong including: Duy1, Viet1, Viet2, Duy2 and Nghia have been collected. These materials give yield from 5.0 to 5.5 kg of cherries/tree, average weight of 100 beans is 16.8 g, ratio of beans on screen 16 ranges from 79.9 to 82.3%. The cup quality scores of these ones ranged from 79 to 81 points (SCA standards). In Lam Dong conditions, those materials have shown completely uninfected with leaf rust.

- Regarding other starting materials grown in Buon Ma Thuot, they have an average yield of 2.3 first harvests over 2.2 tons of beans/ha, weight of 100 beans is >17 g, the ratio of beans on screen 16 is about 80%, the cup quality score is >75, their rust resistance levels are from high to very high.

- Two promising arabica coffee hybrids THA1 x Sr and Sr x THA1 have been selected. These two hybrids have good growth ability; the average yield of the first two harvests is 3.03 and 2.82 tons of beans/ha, 23.2% and 19.7% more comparing to their parent varieties; weight of 100 beans is >17 g; ratio of beans on screen 16 >85%; the cup quality score is 78 points, and they are highly resistant to leaf rust disease.

- Nine outstanding individuals in the F2 generation have been selected including: H32C5, H32C24, H32C29, H32C30, H32C42, H45C122, H45C126, H33C54, H37C86. The yield in the first established year of individuals is from 3.8 to 5.4 kg of cherries/tree, corresponding to the equivalent yield of 2.99 to 4.41 tons of beans/ha [2].

3.2.2. Trial of 03 F1 arabica coffee clones (TN6, TN7, TN9) and the pure line TH1 in suitable ecological regions

- At three trial sites in Lam Dong, Quang Tri, Son La, varieties TN6, TN7 and TN9 grew and

developed quite well; the yield in the harvest season is over 2 tons of beans/ha, in the stable established period, it reaches more than 3 tons of green beans/ha. The weight of 100 beans of TN varieties reached more than 17 g; the percentage of beans on screen 16 is >80%; the cup quality scores of these varieties in Lam Dong reached from 81.50 to 82.75 points, in Quang Tri from 78.50 to 82.25 points, in Son La from 79.50 to 82.50 points. Of which, the TN9 variety in all 3 regions reached over 82 points. In all three regions, TN varieties are highly resistant to leaf rust. Among these three varieties, the TN9 has been tested for distinctiveness, uniformity, and stability (DUS); controlled trial according to basic standards and approved by the scientific committee (decision No. 68/QD-VNLT (SPQG) dated 30/11/2020) and certified by the Department of Agriculture and Rural Development of Lam Dong, Kon Tum, Quang Tri and Son La provinces.

- Regarding the pure line TH1, in 3 trial locations, it grew and developed quite well, yielded from 2.26 to 2.50 in the established year; the average weight of 100 beans is 17.2 g, the percentage of beans on screen 16 ranges from 78.9 - 80.2%; The cup quality scores of TH1 variety in the regions ranged from 82.00 to 84.75 points according to SCA standards. In all 3 areas, this TH1 has shown a light leaf rust infection with the disease index <1% [2].

3.2.3. Building a new breed model applying appropriate farming methods

- Thirty hectares of new arabica coffee varieties have been established in 3 provinces of Kon Tum, Quang Tri and Son La. At the sites where the model is built, these new varieties have shown good growth and wide adaptability. Two hectares of planting model in 2018 in Quang Tri at the established stage give a yield of >3 tons of beans/ha. The average yield of 3 crops was from 2.60 to 2.90 tons of beans/ha, increasing from 22.1% to 36.2% compared to the control. while 28 hectares of planting model in 2019 give the yield 1.66 to 2.19 tons of beans/ha; especially, the TN9 variety in all 3 regions reached over 3 tons of beans/ha, increased from 40.6% to 43.3% compared to the control variety Catimor.

- Economic efficiency of the varieties compared with the control depends on each variety in different growing regions. In Kon Tum, profit from TN varieties varies from 92.5 to 107.2 million VND, increased from 23.8 to 38.5 million VND compared to the control variety. In Quang Tri, the profit of TN varieties ranged from 89.6 to 102.6 million VND, increased from 33.9 to 46.9 million VND compared to the control. In Son La, the profit of TN varieties was from 94.0 to 107.4 million VND, increased from 34.2 to 47.6 million VND compared to the control Catimor. In all 3 regions, the highest rate of increase in profit compared to the control was the TN9 variety with 56.0 to 84.2%, followed by the TN7 variety from 45.7 to 75.3%, the TN6 variety at 34, 6 to 61.0% and only TH1 profit growth rate decreased from 4.0 to 14.0% [2].

3.3. Research and develop synchronous technical packages for coffee to increase the added value of coffee and to serve for domestic consumption and export

3.3.1. Research and perfectionate technical protocols of replanting coffee on the basis of applying integrated solutions focused on mechanization and BAP

- The technical protocol of integrated coffee replanting with mechanization orientation was accredited as a technical advance by the MARD in May 2021. While applying this technical protocol, the production efficiency increased markedly because of increased productivity, decreased water for irrigation and fertilizer uses and saving cost for coffee plantation care and harvesting. Comparing to control, irrigation efficiency increased by over 25%; dissolved fertilizer application through the irrigation system has saved 20 - 40% of fertilizer amount. Coffee planted with high density has significantly increased yield, increasing by more than 50%. Economic efficiency increased by over 90%, equivalent to an increase of over 50 million VND/ha compared to current production [4].

3.3.2. Research to perfectionate the technical protocol of sustainable coffee intensive farming with BAP orientation, pre-harvest and post-harvest processing and improvement of synchronous harvesting equipment to improve production efficiency, reduce costs and post-harvest losses

- The BAP-oriented sustainable intensive farming protocol was evaluated and accepted in December 2020. While applying this protocol, the use of controlled slow-release fertilizers allowed to save about 15% of fertilizer; the use of soluble fertilizer has saved 20% of fertilizer; the quality of green coffee when applying the protocol has been significantly improved compared to mass production. Slow-release fertilizer application has significantly increased yield (increasing by 12.86% for Robusta coffee, increasing by 17.95 - 19.07% for arabica coffee. Economic efficiency of soluble fertilizer application is quite high, more than 10% compared to current production.

- The protocol of pre-harvest and post-harvest processing for coffee has been evaluated and accepted in March 2020. While applying this protocol, the use of ethephon with a concentration of 300 ppm (0.3 liters/tree) spraying at the time of fruit ripening rate of 15 - 20% gave best results. After 20 days of spraying, the percentage of ripe berries was 90.6% (control 54.4%) but the leaf drop rate was 21.95% higher than that of the control 8.45%. It allowed only one picking time that reduced the cost by 32.6% compared to the current harvesting method. It also made the process of mechanized farming and harvesting more convenient. Also, the use of yeast *Saccharomyces cerevisiae* (1 g/kg coffee berries) and *pectinase* enzyme (0.1 g/kg coffee berries) resulted in the best quality of green coffee and cup coffee (cupping score: 72 compared with 58 of the control). It reduced postharvest losses by 4.6%. Moreover, when applying improved coffee harvesting equipment, combined with a mechanized coffee replanting protocol, it saved 34.05% of the cost compared to manual harvesting [4].

3.3.3. Research on building intercropping technical protocol for coffee with BAP orientation

- The protocol of intercropping coffee with increasing production efficiency and adapting to climate change was evaluated and accepted in December 2020. The use rational fertilization for agronomic and economic efficiency showed better results than those of the current production (increasing by 6.5 - 8.1%, corresponding to an increase of 7.6 - 24 million VND/ha). The appropriate amount of irrigation water for first flowering for the intercropping coffee plantation by the method of direct irrigation and mini-sprinkler irrigation has also been determined respectively to be 400 liters/tree and 350 liters/tree [6].

3.3.4. Building 4 models of applying synchronous technical package for sustainable farming BAP orientation (2 models of arabica coffee, 2 models of Robusta coffee, scale: 10 ha/ model)

- Four demonstration models (10 hectares/ model) using newly accredited coffee varieties with high yield and quality applying a synchronous technical package for sustainable farming towards BAP in the Central Highlands and Northwest region. The coffee model in Dak Nong showed an increase of economic efficiency by over 14% and productivity by over 11% compared to current mass production while the one in Dak Lak increases the economic efficiency by over 10% and productivity by over 15%. The arabica coffee model in Dak Lak increased economic efficiency by over 13% and productivity by over 14% compared to mass production while in Son La, increases of 10% for economic efficiency and productivity were recorded [2, 3, 5].

3.4. Producing new high-quality seeds and seedlings for new planting and replanting of coffee in key concentrated regions

3.4.1. Improvement of cultivation and production technical protocols for new high-quality coffee seeds and seedlings

Synthetic improvement of technical measures in this project allowed to build four technical protocols: Cultivation of garden producing hybrid seeds and that of garden of the leading coffee clones; protocol of producing, harvesting,

processing, and preserving coffee seeds; and protocol of producing disease-free and high-quality coffee seedlings. These protocols are accredited by the MARD according to Decision No. 73/QD-TT-CCN dated April 20/2021; moreover, the technological protocol of breeding high-quality coffee by *invitro* methods was accredited according to Decision No. 96/QD-TT-CCN dated May 7, 2021. These protocols are widely applied in production practice, contributing to improving the quality of coffee seeds and seedlings, providing farmers to replant coffee in the main growing areas [6].

3.4.2. High quality planting materials supply

This project has produced seeds and seedlings to supply to growers from 2018-2020 as follow: 5,238 kg of TRS1 seeds; 200,013 grafted coffee trees with TR14 and TRR15 varieties; 2,805,405 coffee trees with TRS1 varieties; 200,230 real coffee and tea plants like THA1; 2,737,000 scalloped coffee plants like TRS1 and 23,490 coffee trees with green beans by tissue culture method like TR4 and TR11. Seedlings and seeds have been produced to provide coffee farmers and coffee growers in the Central Highlands to new/replant coffee with an estimated area of 13,000 - 15,000 ha [5].

3.4.3. Sustainable farming model building

Sustainable farming models using new high-quality coffee varieties have been built in many localities with a total scale of 30 hectares (20 hectares of Robusta coffee with 2 varieties of TR14, TR15 (late ripening) and 10 hectares of arabica coffee with the variety THA1). In all models, these new varieties have shown the ability to grow and adapt widely in the growing areas [5].

4. SHORTCOMINGS, GAPS IN SCIENTIFIC AND TECHNOLOGICAL RESEARCH AND LESSONS LEARNED

- All tasks are fully implemented following the project proposal. However, due to the requirements of the Law on Cultivation, 01 variety of arabica coffee could not been accredited for circulation. Moreover, even 02 varieties of Robusta coffee have been accredited for circulation but this is an exceptional accreditation, and the application scope is narrow in only 03 provinces.

- A total of 14 technical protocols have been evaluated and accredited, including 02 technical advances, 03 ministerial-level protocols. These protocols were all proven to be effective in production. However, it is still necessary to continue to monitor and research to perfectionate a few techniques in the protocols to achieve higher efficiency such as pruning to shape, increasing the rate of mechanization in harvesting, etc.

5. PRODUCT RESEARCH AND DEVELOPMENT ORIENTATIONS TO THE YEAR 2030

- Better cup quality coffee varieties with drought tolerance and uniform ripening traits: to have better coffee quality as national product, breeding research should be prioritized and continued based on starting materials and promising clones inherited from previous period research results. At the same time, varieties with drought tolerance and uniform ripening characteristics can be an important solution to cope with challenges that will face coffee production in the near future.

- From previous period, high quality varieties have been accredited, it is necessary to encourage the expansion of these new varieties in specialized farming areas. At the same time, it is necessary to continue to research and evaluate the interaction between the growing area (climate, soil) with the specific traits of these varieties (yield, quality, drought tolerance), combining sustainable farming methods appropriate to the region to have basis for determining the structure of high-quality varieties for each cultivation area, meeting the demand for specialty coffee production in each specific region.

- We should continue to research and perfectionate the design and manufacture of equipment that can harvest, clean and sort coffee with automatic, highly synchronous mechanization in farming to further improve the efficiency of the protocol and to create high-quality green coffee products.

- We should coordinate with coffee production, processing, and export enterprises in transferring high-quality coffee varieties and applying new technical packages to improve coffee quality, promote deep processing in order to improve coffee quality and to diversify products towards high quality, meeting the objectives of the National Coffee Product Program.

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