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Application of loop-mediated isothermal amplification to detect *Salmonella* spp. in egg samples

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ABSTRACT

The objective of the study was to evaluate the use of loop-mediated isothermal amplification (LAMP) technique for detection of *Salmonella* spp. in chicken eggs. Eight *Salmonella* strains and 26 non-*Salmonella* enteric strains were used to verify the specificity of the LAMP assay for detection of *Salmonella*. The Sal4 primer set was used to specifically amplify the target sequence on the *invA* gene of *Salmonella* and the hydroxyl naphthol blue was used as the indicator to read the reaction results. Blank egg samples were spiked with serial 10-fold diluted *Salmonella* suspensions to determine the limit of detection at 50% (LOD_{50}) of the assay. Egg samples collected from multiple locations in Ho Chi Minh City were tested for the presence of *Salmonella* using both LAMP method and the traditional culture method to compare the effectiveness of these two methods. Among 34 bacterial strains tested by LAMP assay, false-positive or false-negative results were not observed. The assays of spiked egg samples showed that the LOD_{50} of the LAMP method was less than 2 CFU/25 g sample. These results indicated a high specificity and sensitivity of the LAMP assay in detection of *Salmonella* in egg samples, even with those of low levels of contamination. Upon testing collected egg samples (n = 42), the LAMP assay produced the same results of *Salmonella* detection as the culture method. *Salmonella* was detected in 4 out of 42 samples. This study showed that the LAMP method is highly effective and would be suitable for use in detection of *Salmonella* in egg samples. The assay has several advantages such as saving time and labor compared to traditional culture methods.

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1. Introduction

Non-typhoidal *Salmonella* is an important zoonotic pathogen relating to food and feed safety concerns. In 2017, there were an estimation of 95.1 million cases of enterocolitis caused by this pathogen worldwide, with 50.771 deaths and 3.1 million cases of disability-adjusted life-years (Stanaway et al., 2019). Most cases of *Salmonella* infection in humans have been associated with the consumption of contaminated eggs, poultry, pork, beef or dairy products (Park et al., 2014; EFSA & ECDC, 2021). Among them, eggs and egg products are the most important source of *Salmonella* infection (EFSA & ECDC, 2021).

To ensure food safety, it is important to use reliable and effective methods of testing for the presence of *Salmonella* before delivering foods to markets. Usually, *Salmonella* is present in food in low quantities (< 100 CFU/g) along with millions of other bacteria in the sample (Velusamy et al., 2010). Therefore, to detect this bacterium, it is necessary to use a method that has high sensitivity and is not affected by inhibitors in samples.

The conventional culture method instructed by FDA (FDA, 2015) and ISO (ISO, 2017) is recognized as the "gold standard" for the detection of this bacterium in foods. However, this method is labor-intensive, and time-consuming, thus reducing the shelf life of tested foods due to inspecting time (Lin et al., 2020).

Loop-mediated isothermal amplification (LAMP) assay was first introduced by a Japanese research group in 2000 (Notomi et al., 2000). Briefly, in a LAMP assay, 2 - 3 pairs of primers are designed to specifically detect 6 - 8 regions of target gene by using a DNA polymerase with high strand displacement activity (Nagamine et al., 2002). The assay has an efficiently amplified property, which could produce 10^9 copies of the amplified DNA within an hour (Notomi et al., 2000). The technique has been proved to have various advantages such as simplicity, carrying out reactions in an isothermal condition, requiring little of equipment, low cost of chemicals, short processing time, and directly observing of the results by the naked eye (Notomi et al., 2015). Nevertheless, LAMP primers have been said to be a key factor determining success to the assay. Recently, FDA has approved a new set of primers which have been used for detection of *Salmonella* from a variety of food samples including cantaloupe, beef, tomato, etc. (Yang et al., 2016) and animal feeds (Domesle et al., 2020) but eggs. Therefore, this study was conducted to test the applicability of LAMP using these primers to detect *Salmonella* in egg samples.

2. Materials and Methods

2.1. Bacterial strains and culture conditions

The strains used in this study were divided into 2 groups of *Salmonella* (8 strains) and non-*Salmonella* enteric bacteria (26 strains). The strains were either kindly provided by VPH Lab (Faculty of Animal Science and Veterinary Medicine, Nong Lam University HCMC) or isolated from chicken fecal samples in this study (Table 1).

Isolation of *Salmonella* was conducted following instructions by VS (2017). All *Salmonella* strains were confirmed by a slide agglutination test with *Salmonella* O polyvalent antisera (Pasteur Institute, HCMC).

Non-*Salmonella* strains were isolated by culturing freshly collected fecal samples from farm chickens onto MCA agar (MacConkey Agar; Oxoid, CM0115) and/or EMB agar (Eosin Methylene Blue Agar; Oxoid, CM0069). These isolates were roughly identified by the IMViC tests and kept in TSB (Tryptone Soya Broth; Oxoid, CM0129) containing 30% glycerin at -20°C for later use.

2.2. Bacterial enrichment and DNA extraction

Bacterial strains were recovered from frozen stocks by streaking onto MCA plates and incubated at 37°C/24 h. One typical colony of strain was transferred onto a TSA slant (Tryptone soya agar; Oxoid, CM0131) and incubated at 37°C/24 h, which was then kept in a refrigerator during the study. For DNA extraction, a loop of bacteria on TSA slant of each strain was transferred into a 1.5 mL Eppendorf tube containing 500 µL of purified NFW (nuclease-free water; Promega, P1197), and vortexed thoroughly to obtain a homogeneous suspension. The sample was centrifuged at 3000 rpm/10 min and the supernatant was gently discarded (washing step). Then, 200 µL of NFW was added, and the precipitate was dissolved by pipetting. The tube was heated in a water bath at 96°C/10 min, then placed on ice for at least 20 min, and centrifuged at 14000 rpm/10 min. Finally, 100 µL of the supernatant solution (containing DNA) was transferred to a sterilized Eppendorf tube. Bacterial DNA templates were used immediately or stored in a refrigerator for use within 2 - 3 days.

For DNA extraction from egg samples, yolk and white of eggs were pooled into a clean plastic bag, and homogenized by a steriled glass stick. For each sample, 25 g of egg blend was pre-enriched with 225 mL BPW (Buffered Peptone Water; Himedia, RM001) at 37°C for 16 h. One mL of pre-enrichment sample in BPW was transferred to a sterilized 1.5 mL tube and centrifuge at 3000 rpm/10 min. The supernatant was discarded followed by adding 500 µL of NFW and vortexed thoroughly. Then, samples were washed and extracted DNA as described above.

2.3. *Salmonella*-LAMP assay

The Sal4 primer set (Table 2) and the thermal condition of reactions were adapted from Yang et al. (2016). LAMP reactions were carried out in 200 µL Eppendorf tubes with a total volume

Table 1. Bacterial strains (n = 34) used for optimization of the *Salmonella*-LAMP assay[1]

Species/groups	Origin	Numbers of strains	Codes
Salmonella strains	VPH lab, Nong Lam University HCMC	4	NLS001, NLS002, NLS003, NLS004
	From chicken fecal samples in this study	4	NLS005, NLS006, NLS007, NLS008
E. coli strains	VPH lab, Nong Lam University HCMC	1	NLE001
	From chicken fecal samples in this study	9	NLE002 to NLE010
Coliforms	From chicken fecal samples in this study	10	NLC001 to NLC010
Proteus spp.	From chicken fecal samples in this study	3	NLP001 to NLP004
Shigella spp.	From chicken fecal samples in this study	3	SHI001 to SH003

[1]LAMP: loop-mediated isothermal amplification.

of 20 µL including the following reactants: 2 µL of IAB II, 10X (Isothermal Amplification Buffer; New England Biolabs, M0538S); 1.2 µL $MgSO_4$ (100 mM); 0.8 µL BST (*Bacillus stearothermophilus*) 3.0 polymerase (8000 U/mL) (New England Biolabs, M0538S); 2.4 µL dNTP Mix (25 mM) (New England Biolabs, N0447S); 1.5 µL dimethyl sulfoxide; 1.8 µM FIP/BIP primer; 0.1 µM primer F3/B3; 1.0 µM Loop-F/B primer (Integrated DNA Technologies); 1.0 µM HNB (300 mM) (hydroxy naphthol blue; Alpha Chemika, 63451-35-4), 2 µL of sample DNA extraction and added NFW to 20 µL. The reactions were performed at 65°C for 45 min using a PCR machine (Benchmark, TC-32). The reaction was stopped at 80°C/10 min.

The result of the LAMP reaction was read based on the color change of the hydroxy naphthol (HNB) indicator. Positive results were obtained when the reaction turned blue color; while, negative samples still remained violet color (Goto et al., 2009).

2.4. Determination of specificity of the *Salmonella*-LAMP

The specificity of the *Salmonella*¬-LAMP assay was performed with 8 strains of *Salmonella* spp. and 26 strains of non-*Salmonella* bacteria (Table 1). The specificity results of the LAMP assay were verified by the number of reactions that gave positive results with *Salmonella* strains and the number of reactions that gave negative results with non-*Salmonella* strains. In addition, to interpretation of the color change of each reaction, the LAMP products were electrophoresed on 1.5% agarose gel to confirm the results.

2.5. Determination of sensitivity of *Salmonella*-LAMP

To prepare blank samples, the yolk and white of each egg were homogenized by a sterilized glass stick and 25 g of the egg blend was taken for detection of *Salmonella* by culture following the instructions of VS (2017). Samples from which *Salmonella* was not detected were used as negative control, which were stored in refrigerator at 4°C for using within 3 to 4 days. The suspensions of *Salmonella* strains NLS001 and NLS002 were serial 10-fold diluted to concentrations of 10^0 - 10^4 CFU/mL. One mL of each bacterial dilution was spiked into each of 5 blank samples (25 g egg blend/sample), the suspension was homogenized by shaking sample bags. Each spiked sample was added 225 mL PBW and incubated at 37°C/24 h. DNA was extracted and *Salmonella*-LAMP reactions were performed. The limit of detection at 50% (LOD_{50}) was determined following ISO guidelines (ISO, 2016).

2.6. Detection of *Salmonella* by LAMP versus culture method

Commercial eggs were purchased from 7 locations that are mini-supermarkets and 7 locations that are individual business households at wet markets in Ho Chi Minh City for *Salmonella* de-

Table 2. The Sal4 primers set for *Salmonella*-LAMP[1]

Primers	Sequence (5' – 3')	bp
FIP	GCGCGGCATCCGCATCAATATCTGGATGGTATGCCCGG	38
BIP	GCGAACGGCGAAGCGTACTGTCGCACCGTCAAAGGAAC	38
F3	GAACGTGTCGCGGAAGTC	18
B3	CGGCAATAGCGTCACCTT	18
Loop-F	TCAAATCGGCATCAATACTCATCTG	25
Loop-B	AAAGGGAAAGCCAGCTTTACG	21

[1]Yang et al. (2016); LAMP: loop-mediated isothermal amplification.

tection by using both conventional culture and *Salmonella*-LAMP methods. Samples from ten eggs were pooled as one sample for analysis. At each site, three pooled samples were collected. In this study, total 42 pooled samples were used for detecting the presence of *Salmonella*. To collect the egg content, egg shells were disinfected with 1% iodine alcohol solution for 1 minute and dried at room temperature. Ten disinfected eggs were cracked, pooled into a clean plastic bag, and homogenized by a steriled glass stick. An amount of 25 g of each sample was pre-enriched with 225 mL BPW at 37°C/16 - 24 h. Pre-enriched samples were analyzed for *Salmonella* by both LAMP assay as described in sections 2.2 and 2.3 and culture method as described in VS (2017). Results of *Salmonella* detection from both methods were compared.

3. Results and Discussion

3.1. Specificity of the *Salmonella*-LAMP

All 8 *Salmonella* strains gave positive results with the LAMP assay whereas no color change (negative results) was observed from all 26 samples of non-*Salmonella* strains. This agreed with the summary of the meta-analysis study by Yang et al. (2018) of which many LAMP reactions used for detecting different *Salmonella* strains had specificity rates of 97.4 – 100% with positive control samples, and 100% with negative controls. When electrophoresis was conducted for LAMP products of the control strains, all 8 *Salmonella* strains showed ladder-like pattern of LAMP products and all non-*Salmonella* samples did not have the presence of LAMP products (Figure 1).

3.2. Sensitivity of *Salmonella*-LAMP

Limit of detection at 50% obtained by the *Salmonella*-LAMP on egg samples was less than 2 CFU/25 g (Table 3). This agreed with the results of 10^0 CFU/25 mL by Yang et al. (2013), and 1.63 - 4.18 CFU/25 g by Hu et al. (2018). Meanwhile, LOD_{50} obtained by the traditional culture method on the dried egg powder samples (with confidence interval 95%) was 6.0 (4.7 - 7.7) CFU/25 g (the annex attached to ISO (2017) for detecting *Salmonella*). Thus, *Salmonella*-LAMP showed a better detection of *Salmonella* at low levels of contamination in samples comparing to the culture method.

3.3. Efficacy of LAMP assay in detection of *Salmonella* from egg samples

3.3.1. Detection of *Salmonella* in egg samples

A total of 42 pooled egg samples were tested for the presence of *Salmonella* by both LAMP and culture methods, from which the results were similar (Table 4). This would indicate a strong recommendation to use LAMP for detection of *Salmonella* in egg samples over the culture method.

The high similarity in results of *Salmonella* detection by the two methods have been noted in many studies (Zhang et al., 2011; D'Agostino et al., 2016; Hu et al., 2018). Wang et al. (2015) showed similar results obtained by LAMP and culture without and with a pre-enrichment step (89.58% and 100%, respectively). In a recent study (Ge et al., 2019), the comparison of LAMP and culture methods to detect *Salmonella* in multiple laboratories concluded that LAMP method rigorously met the validation of AOAC, FDA, and ISO guidelines. Ge et al. (2019) also proposed the use the LAMP assay to screen samples for *Salmonella* and only those with positive results

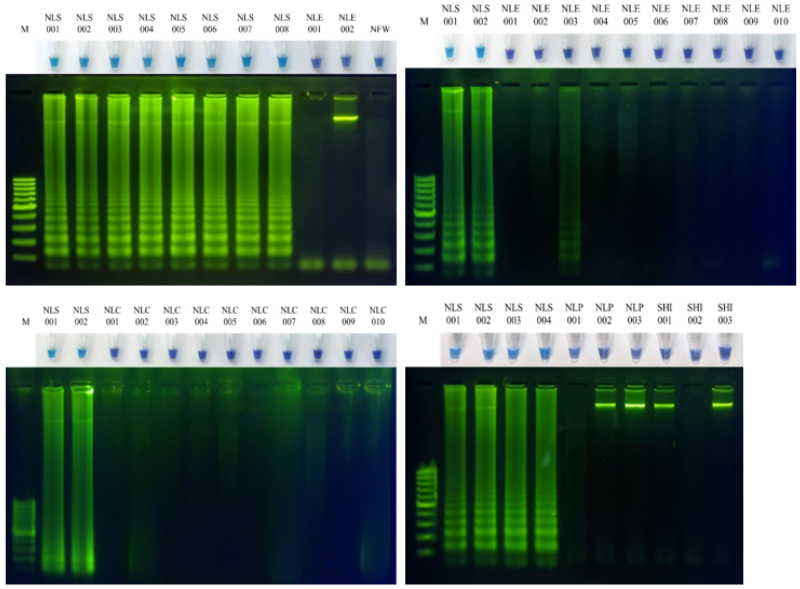

Figure 1. Results of *Salmonella*-loop-mediated isothermal amplification were obtained by hydroxy naphthol blue indicator and electrophoresis. Lane M, 100 bp DNA ladder (ThermoFisher, 15628019); Lane NFW, nuclease-free water; Lanes NLS: *Salmonella* strains; and others were non-*Salmonella* strains.

Table 3. Limit of detection of *Salmonella*-loop-mediated isothermal amplification for egg samples spiked with NLS001 and NLS002 strains

NLS001		NLS002	
Concentration	Positive	Concentration	Positive
0 CFU/25g	0/1	0 CFU/25g	0/1
0.95×10^0 CFU/25 g	4/5	1.14×10^0 CFU/25 g	3/5
0.95×10^1 CFU/25 g	4/5	1.14×10^1 CFU/25 g	5/5
0.95×10^2 CFU/25 g	5/5	1.14×10^2 CFU/25 g	5/5
0.95×10^3 CFU/25 g	5/5	1.14×10^3 CFU/25 g	5/5
LOD_{50} (*) (95% confidence interval)	1.91 (0.64 - 5.65)	LOD_{50} (*) (95% confidence interval)	0.86 (0.26 - 282)

(*): LOD_{50} or limit of detection at 50% and 95% confidence interval calculated following the guidance of ISO (2016).

Table 4. Comparison of *Salmonella* detection in egg samples using the *Salmonella*-LAMP (loop-mediated isothermal amplification) and culture methods

		Culture	
		Positive	Negative
LAMP	Positive	100% (4/4)	0%
	Negative	0%	100% (38/38)
	Total	4	38

should undergo bacterial isolation. This would reduce time, labor, and increase the efficiency of testing and isolating *Salmonella* from samples.

Salmonella was detected from 4 of 42 egg samples (9.5%), of which 3 samples were from mini-supermarkets and 1 sample was from a wet market. This was higher than those from previous reports. For example, the bacteria were found from 2 out 52 egg yolk samples taken in Hau Giang province (Tran, 2012), while the bacteria were not detected from 115 egg samples collected from some retailers in Ha Noi (Ha et al., 2017).

Meanwhile, high detection rates (at over 25%) of *Salmonella* from poultry, pork and beef samples have been reported (Luu et al., 2013; Le et al., 2019). In a study in Ho Chi Minh City, 68.4% meat samples were detected with *Salmonella* (Nguyen et al., 2018). These results strongly indicate high prevalence of *Salmonella* presence in products of animal origins in Vietnam.

A report in Europe in 2020 presented low prevalence of *Salmonella* in egg and egg products (0.63%) and in fresh meat samples of all kinds (0.40 - 8.01%) (EFSA & ECDC, 2021). However, outbreaks of human salmonellosis in Europe have been most associated with the consumption of eggs and egg products. In the detail, 44% of 84 salmonellosis outbreaks in Europe in 2020 were associated with eggs and egg products, followed by pork and pork-related products with only 13.1% (EFSA & ECDC, 2021). This shows that although the prevalence of *Salmonella* in eggs is lower than in meat products, the role in food safety of this bacterial contamination in egg samples is of significant importance. Therefore, the high prevalence of *Salmonella* in eggs in this study represents a major health hazard to consumers.

3.3.2. Efficiency of the *Salmonella*-LAMP assay

The LAMP assay for detection of *Salmonella* consumed approximately 20 h, of which most of the time (16 h) was spent on the pre-enrichment phase. Techathuvanan et al. (2012) noted that the limit of detection of *Salmonella* by LAMP for pre-enriched samples at 6 h, 12 h, & 16 h were 10^6, 10^4, & 10^0 CFU/25 mL, respectively. Therefore, a pre-enrichment step for 16 h would be optimal to obtain reliable results, especially with samples that have low levels of contamination. In other words, it would take less than a day to get the results for *Salmonella* detection in food samples using LAMP assay comparing 5 - 7 days by the standard culture method. LAMP is more suitable for testing *Salmonella* in products having a short shelf life such as eggs and ready-to-eat foods (Lin et al., 2020). Furthermore, the assay meets the need of quickly testing pathogens in hatched eggs for breeding in poultry husbandry (Trampel et al., 2014).

4. Conclusions

The *Salmonella*-LAMP assay demonstrated advantages including convenience, simplicity in chemical preparation, and saving time and labor when compared with the standard culture method. Therefore, it would be strongly recommended as an alternative approach for detection of *Salmonella* in egg samples.

Conflict of interest

The authors have no conflicts of interest to declare.

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Efficiency of intensive tilapia culture in earthen ponds applied biofloc technology (BFT), probiotics and off-flavor control methods

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ABSTRACT

The aim of this study was to assess the efficiency of Nile and red tilapia cultured in intensive systems applied a combination of biofloc technology (BFT) and probiotics in earthen ponds with different methods to control the off-flavor problem. A growing-out experiment was conducted for 150 days in which both Nile tilapia (5.88 ± 0.59 g) and red tilapia (9.72 ± 0.72 g) fingerlings were randomly allotted into three replicates of earthen ponds which were subdivided into four treatment groups, including: off-flavor controlled by algae management in Nile tilapia ponds (NT - AM) and red tilapia ponds (RT - AM), and off-flavor controlled by active water exchange in Nile tilapia ponds (NT - WE) and red tilapia ponds (RT - WE). The results showed that water quality was maintained in suitable ranges for fish growth. There were no significant differences in growth performance (final weight, daily weight gain and specific growth rate) among all treatments ($P < 0.05$). Feed conversion ratio of Nile tilapia (1.28 - 1.31) was significantly lower than that of red tilapia (1.35 - 1.37) ($P < 0.05$). The survival ratio (about 80%) and extrapolated yield (21.50 tons/ha in 150 days) of fish was similar and high for both Nile and red tilapia. The quality of the harvested fish in term of condition factor, size even and off-flavor intensity was also excellent. The economic efficiency of red tilapia farming in this system was higher as compared to cage systems. This study clearly demonstrated that the technique of combined BFT and probiotic application in earthen ponds could contribute to the sustainable development of tilapia production in Vietnam by reducing production cost, saving water resource and avoiding environmental pollution.

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1. Introduction

Tilapias originate from Africa and the Middle East but now are introduced into many territories and cultivated worldwide, particularly in Asia and the Pacific (De Silva et al., 2004). Tilapias are herbivorous and fed mainly on planktonic organisms, aquatic macrophytes and detritus, and they are able to tolerate low oxygen tension and a wide range of salinity and temperature, and resist to diseases (Ng & Romano, 2013). All tilapia species with important commercial values farming outside Africa belong to *Oreochromis* genus and more than 90% is Nile tilapia (*Oreochromis niloticus*) (Watanabe et al., 2002). In 2018, tilapias were the second species of cultured food-fish, after carps, with a production of 4,525.5 thousand tons and accounted for 8.3% of the major aquaculture finfish (FAO, 2020).

Early introduced to Vietnam since 1950s, tilapias have commonly raised in mono - and polyculture systems in earthen ponds and cages

in both freshwater and brackish water environments with different intensification levels. So far, Nile tilapia strains have been commonly cultured in ponds (Nguyen et al., 2004; Nguyen et al., 2006) and red tilapia (*O. niloticus x O. mossambicus*) strains in cages (Phan, 2015; Tran, 2016). In 2015, total tilapia production was 187,000 tons with a total value of 200 million USD. In 2017, tilapia product was exported to 68 international markets with a revenue of 45 million USD, an increase of 32% compared to 2016 (GSO, 2021). Tilapia production was also planned to increase and oriented toward export in the future (MARD, 2016).

According to Yue et al. (2016), land-based recirculating aquaculture, and cage and offshore aquaculture can help reduce the negative impacts of tilapia culture on the environment and global biodiversity. One of problems of tilapia cultured in earthen ponds is the development of off-flavor (Fitzsimmons, 2008). Tran (2016) noted that water quality in areas of red tilapia culture in cages in the Mekong River Delta (MRD) has declined, leading to increased disease incidence, mortality and feed utilization. Recently, several modern technologies, new approaches and alternative methods, including probiotics, have been applied to improve the production as well as quality of Nile tilapia (Aly et al., 2008). The biofloc technology (BFT) combines the removal of nutrients from the water with the production of microbial biomass, which can *in situ* be used by the cultured species as additional food source (De Schryver et al., 2008). Aquaculture using these technologies offers aquaculture a sustainable tool to simultaneously address its environmental, social and economic issues concurrent with its growth (Crab et al., 2012). In Vietnam, farming tilapia with BFT has been implemented in tanks (Nguyen, 2012; Le et al., 2016) and in concrete ponds (Nguyen et al., 2013). Phan et al. (2021) was successful in combined application of BFT and probiotics in Nile tilapia culture in earthen ponds. This experiment aimed to assess the ability of combination of BFT, probiotic and off-flavor control application in intensive tilapia culture in earthen ponds, as an alternative solution for red tilapia culture in cages.

2. Materials and Methods

Study site

The study was carried out in Long Xuyen city of An Giang province in the MRD of Vietnam from May to December, 2020.

Experimental design

The experiment was set up as a completely randomized design with four treatments to assess the efficiency of tilapia strains cultured intensively in earthen ponds. The combination of BFT and probiotic use but different off-flavor control methods was applied for treatments as following:

- NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds;

- NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds;

Each treatment was replicated in three 200 m^2 earthen ponds with a water depth of 1.5 m. The ponds were drained, completely removed bottom mud, applied $CaCO_3$ at a dose of 7 -10 kg/100 m^2 and dried for 2 - 3 days. Water was supplied into the ponds using filter bags to eliminate unwanted animals. The ponds then fertilized with urea at a dose of 1 kg/100 m^2 to develop natural feed. Pond water was aerated with air-tubes fixed on round aluminum dishes and convectively moved with air-lift systems (Phan et al., 2021).

All-male fingerlings of GIFT strain of Nile tilapia with initial weights of 5.88 $\pm$ 0.59 g were supplied from the Tilapia Selection Center of the Research Institute for Aquaculture No. 1. The fingerlings of selected red tilapia strain with initial weights of 9.72 $\pm$ 0.72 g were supplied from the National Breeding Center for Southern Freshwater Aquaculture of Research Institute for Aquaculture No. 2. When pond water became green, the fingerlings were immersed in salt water of 2 - 3% for 5 - 10 min to eliminate parasites and stocked at a density of 5 ind/m^2 in the morning. The air-tube and air-lift systems were operated for 24/24 h after fish stocking.

Biofloc, feeding and algae management

Biofloc booter was prepared by mixing 30 g of feed and 30 g of probiotic Pond Plus of Bayer Company with a mixture of *Bacillus* spp. (*B. subtilis, B. megaterium, B. amyloliquefaciens, B. licheniformis and B. pumilus* $\geq 1.0 \times 10^9$ CFU/g) in 3,000 mL clean water. The mixture was aerated and steered for 24 - 48 h at pH of 6.0 - 7.2. The ponds were supplied the biofloc booter at a dose of 5 ppm/day for the first month. In

following months, the ponds were supplied the mixed *Bacillus* spp. probiotics at a dose of 10 g/100 m^2 and at an interval of 10 days. Moreover, molasses (C = 37.5%) as a supplementary carbon source was weekly added to the ponds at a dose of 3.5 g/m^3 of pond water to maintain the C/N ratio of about 11.5/L and stimulate the flocs formation (Nguyen et al., 2013; Phan et al., 2021).

Pelleted feed for tilapia of Green Feed Company was supplied to the fish followed a feeding regime as presented Table 1.

The fish was fed twice a day at 08:00 - 09:00 and 15:00 - 16:00. The feeding was stopped one day per week to stimulate the fish consuming flocs (Nguyen et al., 2013). At thirty-day intervals, a sample of 30 fish individuals of each replicate was randomly collected for size (total length and weight) measurement.

In the NT-AM and RT-AM ponds, no water exchanged was applied but copper sulfate ($CuSO_4.5H_20$) was used at a dose of 2 kg/ha pond to kill algae when transparency dropped below 30 cm then *Bacillus* spp. probiotics was applied to improve water quality. In the NT-WE and RT-WE ponds, active water exchange was applied during the last month of the cultivation to maintain the transparency $\geq$ 30 cm.

Water quality monitoring

Water temperature, dissolved oxygen (DO) and pH were measured in the morning (06:00) and afternoon (15:00) using portable DO and pH meters of HANNA Company at a 3 days interval. Transparency and ammonia were measured weekly using secchi dish and indophenol blue method (APHA, 1995). At the end of the experiment, a pooled water sample of the ponds was primarily tested for quality parameters (pH, biological oxygen demand, chemical oxygen demand, total suspended solids and *Coliform*) (APHA, 1995). These parameters were assessed for waste water quality followed the National Technical Regulation No. 02-26:2017/MARD (MARD, 2017).

Production efficiency analysis

The fish growth, feed utilization, product quality and economic efficiency were assessed as follows:

- The specific growth rate (SGR) (Mehrara et al., 2009):

Table 1. Feeding regime and pelleted feed (Green Feed company) given to tilapia in the experiment

Crude protein (%)	35			30			25		
Feeding rate (% BW)	7	5	4	3		3	2.5		2
Weeks	1 - 2	3 - 4	5 - 8	9 - 10		11 - 12	13 - 16		17 - 20

SGR (%/day) = 100*(LnW_f – LnW_i)/T, where W_i = initial fish weight (g), W_f = final fish weight (g), T = experiment period (day) and Ln = natural logarithm.

- The feed conversion ratio (FCR) (De Silva & Anderson, 1994)

FCR = $F_c/(M_f - M_i)$, where F_c = total feed consumed by fish (kg), M_i = total fish weight at beginning (kg) and M_f = total fish weight (kg) at the end of the experiment.

- Assessment of off-flavor of the harvested fish followed Fitzsimmons (2008): fillets of three randomly sampled fish of each replicate were wrapped in aluminum foil and steamed in a microwave oven for 60 sec. Off-flavor intensity of the fillets was sensuously assessed by a judge of nine untrained persons based on a five-level scale with 1 = very strong and 5 = no off-flavor. Flavor intensity was expressed by the average of level scores of the judge.

- Economic efficiency was estimated followed Do (2010) based on total cost and revenue for 1 ha/crop as following: benefit = total revenue – total cost, and capital efficiency (benefit-cost ratio, BCR (%)) = 100*benefit/total cost.

Statistical analysis

All experiment data were statistically analyzed by one-way analysis of variance (ANOVA), using Tukey's post hoc ANOVA test for individual comparisons ($P < 0.05$, level of significance). All statistical analyses were carried out by using the Minnitab software version 16.0 program.

3. Results and Discussion

3.1. Water quality

There were no significant differences in the water parameters among the ponds (Table 2). The mean transparency of the ponds was low during the experiment, hence implying high density of algae. The temperature, pH and DO average values in the afternoon were higher than in the morning related to the photosynthesis and respiration of the algae. Ammonia was maintained at low concentrations.

3.2. Growth performance

The growth performance in terms of total length and weight of the fish in all treatments during the cultivation was quite consistent and

Table 2. Average values of water quality parameters of the experimental ponds

Water parameters	Time	Treatments[1]			
		NT-AM	NT-WE	RT-AM	RT-WE
Temperature (°C)	Morning	25.50 ± 2.19	25.41 ± 2.15	25.72 ± 2.16	24.61 ± 2.15
	Afternoon	28.32 ± 2.18	28.59 ± 2.12	28.62 ± 2.19	28.45 ± 2.18
pH	Morning	7.20 ± 0.10	7.16 ± 0.08	7.18 ± 0.10	7.18 ± 0.08
	Afternoon	7.82 ± 0.10	8.78 ± 0.09	7.80 ± 0.09	7.82 ± 0.09
Dissolved oxygen (mg/L)	Morning	5.22 ± 0.33	5.18 ± 0.35	5.20 ± 0.36	5.18 ± 0.35
	Afternoon	6.25 ± 0.34	6.24 ± 0.39	6.26 ± 0.37	6.27 ± 0.39
Ammonia (NH_3-N) (mg/L)		0.08 ± 0.02	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02
Transparency (cm)		26.70±1.70	26.70 ± 1.70	26.70 ± 1.70	26.70 ± 1.70

[1]NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds; NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds.

in a same trend (Figures 1 and 2). In general, the length of the fish showed a high increase in the first ninety days but slow in the last sixty days. Different from length growth, the increase in body weight was low in the first sixty days but high thereafter.

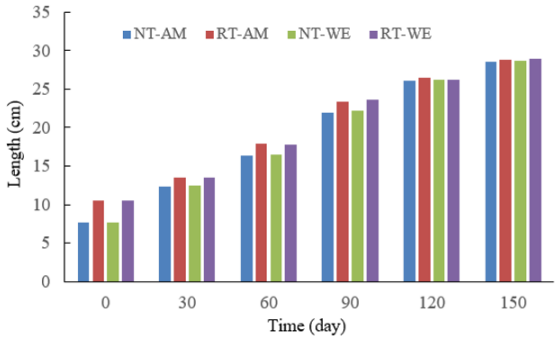

Figure 1. Growth in total length of the fish during culture period. NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds; NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds.

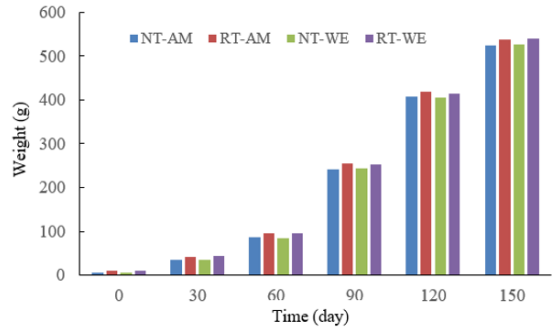

Figure 2. Growth in weight of the fish during culture period. NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds; NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds.

Although the initial weight of the stocked fingerlings of red tilapia was higher than that of Nile tilapia, there were no significant differences in the means of total harvested biomass among the treatments ($P > 0.05$). The total consumed feed, average feed intake (AFI) and feed conversion ratio (FCR) of red tilapia were significantly

Table 3. Growth performance of the experiment fish

Parameters	Treatments[1]			
	NT-AM	NT-WE	RT-AM	RT-WE
Final weight (g)	523.50 ± 2.09	526.72 ± 3.50	538.39 ± 7.66	539.78 ± 8.34
Daily weight gain (g/day)	$3.45^a \pm 0.01$	$3.47^a \pm 0.02$	$3.52^a \pm 0.05$	$3.53^a \pm 0.06$
Specific growth rate (%/day)	$2.99^a \pm 0.00$	$3.00^a \pm 0.00$	$2.68^a \pm 0.01$	$2.68^a \pm 0.01$

[1]Means within the same row with different superscript letters are significantly different at $P < 0.05$ where a < b; NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds; NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds.

higher than those of Nile tilapia ($P < 0.05$), but no significant difference was found between any these values of the same fish strains ($P > 0.05$) (Table 4).

Survival ratio (SR) of the fish were high and not significantly different ($P > 0.05$), which resulted in high extrapolated yields. Significantly lower coefficient of variation (CV) expressed an even weight size of the harvested fish in the NT-AM ponds compared to the others ($P < 0.05$). High scores pointed out the good quality in terms of off-flavor intensity of all treatments (Table 5).

The quality in terms of pH, BOD, COD, TSS and *Coliform* of the effluent after fish harvesting of the ponds responded the Vietnam's criteria to be directly discharged into the environment (Table 6).

Economic efficiency which was estimated for red tilapia of the RT-AM treatment extrapolated to 1 ha for five-month cultivation as a demonstration is presented in Table 7. Feed cost occupied the highest ratio of the production cost, followed by energy, probiotics and labor costs. The production cost was estimated about 27,900 VND. With a farm price of 32,000 VND/kg, The net benefit was 88,136,4000 VND with a ratio of benefit/cost was 14,69%.

4. Discussion

Cruz et al. (2013) proposed that the use of probiotics is recommended in culture system with high concentrations of produced nitrogen compounds, especially the highly toxic total ammonia, to improve water quality. The ammonia (NH_3-N) concentrations in this experiment (0.04 - 0.05 mg/L) were lower than those of tilapia intensively cultured in earthen ponds without BFT (0.36 - 0.70 mg/L) (Nguyen et al., 2006) and in concrete ponds with BFT and no probiotic application (0.21 - 0.27 mg/L) (Nguyen et al., 2013). According to Dauda et al. (2013), improved water quality was particularly associated with *Bacillus* spp. in aquaculture. Zhou et al. (2010) found that the separate application of probiotics of *Bacillus subtilis, B. coagulans* and *Rhodopseudomonas palustris* in water had no improvement of water quality in Nile tilapia culture in recycled water tanks. The water quality of the ponds in this study were further improved compared to that of the tilapia BFT ponds (Nguyen et al., 2013)

Table 4. Feed utilization of the experiment fish

Parameters	Treatments[1]			
	NT-AM	NT-WE	RT-AM	RT-WE
Total stocked fish biomass (kg)	5.88 ± 0.59	5.88 ± 0.59	9.72 ± 0.72	9.72 ± 0.72
Total harvested fish biomass (kg)	422.99^{a} ± 3.08	430.14^{a} ± 0.23	430.52^{a} ± 6.18	433.23^{a} ± 3.90
Total consumed feed (kg)	544.69^{a} ± 2.94	544.84^{a} ± 3.67	576.23^{b} ± 5.06	573.24^{b} ± 5.02
Average feed intake (g)	674.13^{a} ± 3.65	667.17^{a} ±4.50	720.62^{b} ± 6.32	714.21^{b} ± 6.24
Feed conversion ratio	1.31^{a} ± 0.01	1.28^{a} ± 0.01	1.37^{b} ± 0.02	1.35^{b} ± 0.02

[1]Means within the same row with different superscript letters are significantly different at $P < 0.05$ where a < b; NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds; NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds.

Table 5. Culture efficiency, biometric indice and quality of the harvested fish

Parameters	Treatments[1]			
	NT-AM	NT-WE	RT-AM	RT-WE
Extrapolated yield (ton/ha)	21.15	21.51	21.53	21.66
Survival ratio (%)	$80.80^a \pm 0.44$	$81.67^a \pm 0.55$	$79.97^a \pm 0.70$	$80.30^a \pm 0.99$
Coefficient of variation of final BW (%)	$5.65^a \pm 0.49$	$7.82^b \pm 3.41$	$6.77^b \pm 0.25$	$7.35^b \pm 0.86$
Score of off-flavor intensity	$4.70^a \pm 1.06$	$4.92^a \pm 0.19$	$4.85^a \pm 0.77$	$4.96^a \pm 0.27$

[1]Means within the same row with different superscript letters are significantly different at $P < 0.05$ where a < b; NT - AM and RT - AM: off-flavor controlled by algae management in Nile and red tilapia ponds; NT - WE and RT - WE: off-flavor controlled by active water exchange in Nile and red tilapia ponds.

and probiotic applied tanks (Zhou et al., 2010) coming from the combined application of BFT and mixed Bacillus spp. probiotics (Loan et al., 2021). In general, the water parameters in this experiment were within suitable ranges for tilapia growth (Balarin & Haller, 1982).

Many studies have suggestedthat probiotics can serve asa growth promoter for important species in aquaculture (Dawood & Koshio, 2016). In this study, with the same stocking density of 5 fish/m^2, the DWG of Nile tilapia (3.45 - 3.47 g/day) and red tilapia (3.52 - 3.53 g/day) was higher than that of tilapia intensively cultured in the concrete tanks with BFT (2.3 - 2.5 g/day) (Nguyen, 2012) and similar to that of tilapia intensively cultured in concrete ponds with BFT (3.46 - 3.60 g/day) (Nguyen et al., 2013). The growth in terms of SGR of Nile tilapia (2.99 - 3.00%/day) and red tilapia (2.68%/day) was also higher that of tilapia (2.50 - 2.58%/day) cultured in tanks with BFT and different salinities (Le et al., 2016). In the present study, FCR of Nile tilapia (1.28 - 1.31) and red tilapia (1.35 - 1.37) was similar to those of tilapia cultured in concrete ponds (1.33 - 1.37) (Nguyen et al., 2013) and in tanks (1.29 - 1.41) (Le et al., 2016). The lower FCR of Nile tilapia compared to red tilapia indicates the better use of flocs of the former.

There were no significant differences in the SR and extrapolated yield between Nile and red tilapia strains. The SR of the fish in this study (about 80%) was higher than that of red tilapia cultured in cages (69.2%) (Phan, 2015). The extrapolated yield of all treatments (21.15 - 21.66 tons/ha in 150 days) was similar to that of tilapia cultured in concrete ponds with BFT (23.00 - 24.80 tons/ha in 172 days) (Nguyen et al., 2013). In addition to the combination of BFT and probiotic supplement, the methods of algae management and active water exchange further improved the quality of harvested fish in terms of off-flavor intensity (Table 4).

Coldebella et al. (2018) found that the concentrations of TSS, COD and other of organic matters in the pond water of intensive tilapia culture increased significantly at the end of the farming cycle, which caused a progressive deterioration of the environment. The primary analysis of discharged water quality included parameters of pH, BOD, COD, TSS and *Coliform* at the end of the cultivation showed that the combination of BFT and probiotic application resulted in the ful-

Table 6. Quality parameters of pooled pond water after harvesting

Parameters	Unit	Analyzed values	National Technical Regulation No. 02-26:2017/MARD
pH		7.2	5.5 - 9
Biological oxygen demand (BOD_{5-20}°C)	mg/L	39.72	$\leq$ 50
Chemical oxygen demand (COD)	mg/L	85.95	$\leq$ 150
Total suspended solids (TSS)	mg/L	94.7	$\leq$100
Coliform	MPN/100 mL	4,289	$\leq$ 5,000

Table 7. Economic efficiency analysis for red tilapia cultured in the experiment

Parameters	Amount	Value (VND)	Ratio of total cost (%)
Extrapolated yield (ton/ha in 5 months)	21.5		
Total cost		599,863,600	100
Fixed cost (Depreciation of aeration system)		15,000,000	2.50
Variable cost		584,863,600	97.50
Feed (ton)	29,025	440,193,600	73.38
Electricity for aeration, water supply (kW)	17,000	37,400,000	6.23
Probiotics (kg)	21.5	25,800,000	4.30
Permenant labor (1 labor × month)	5	25,000,000	4.17
Others (molasses, Vitamin C, $CuSO_4.5H_20$,...)		56,470,000	9.41
Production cost for 1 kg of red tilapia		27,900	
Total revenue		688,000,000	
Net benefit		88,136,400	
Benefit-cost ratio (%)		14.69	

fillment of the effluent quality after fish harvesting to Vietnam's criteria to be directly discharged into receiving water bodies (Table 5).

Crab et al. (2012) emphasized that in transferring BFT to farmers, the economic benefits of the implementation of this technique was also a very important aspect. The feed and seed cost ratios (73.38 and 0.83, respectively) of red tilapia culture in this study was lower than those (80.04% and 14.67%, respectively) of red tilapia cultured in cages. The high ratio of seed cost in cage systems due to larger fingerlings required (Phan, 2015). The BCR of red tilapia of the experiment (14.69%) was higher than that of red tilapia cultured in cages (11.4%) (Phan, 2015) but lower than that of tilapia cultured in different systems such as in earthen ponds without BFT (18.97%) (Nguyen et al., 2006) and in concrete ponds with BFT (19.17%) (Nguyen et al., 2013). In the case of an increased shortage and degradation of running water, this method could avoid the risk of diseases, thereby save the cost of medicines, increasing the quality of product and contributing to sustainable development of tilapias in general and red tilapia farming in particular.

5. Conclusions

The major goal of the present study was to develop and assess the efficiency of applying a combination of BFT and probiotics with different off-flavor control methods in intensively cultured tilapia in earthen ponds. The results showed that the water quality of this system was maintained within suitable ranges for the growth of fish. The growth performance (DWG and SGR), feed consumption, production efficiency (FCR, SR, yield and quality of the fish) were also improved compared to the separate use of BFT and probiotics. The quality of the harvested fish in term of size even and off-flavor intensity was also excellent. This system brought higher economic efficiency for red tilapia farming compared to cage system. This study clearly demonstrated that the combined application of BFT, probiotic and off-flavor control is an advanced approach for production of high-quality tilapias to support the plan of government for the development of tilapia aquaculture for export.

Conflict of interest

The authors have no conflicts of interest to declare.

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Optimization of enzymatic hydrolysis condition of edible bird's nest crumbs by response surface methodology and determination of biochemical characteristics of the hydrolysate

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ABSTRACT

Edible bird's nest (EBN) crumbs are the by-product of the bird's nest industry. Despite having lower economic value compared to the original material, EBN crumbs still maintain high protein and carbohydrate content. Therefore, this study aimed to determine the optimal hydrolysis condition for EBN crumbs using protease to achieve the maximum degree of hydrolysis (DH). Plackett Burman design was employed to identify the important factors. The results showed that enzyme loading, temperature and hydrolysis time had the strongest effect on the DH. These factors were subsequently subjected to the optimization study using central composite design (CCD) of response surface methodology (RSM). The optimized conditions for the enzymatic hydrolysis of EBN crumbs were at an enzyme loading of 4%, temperature of 51°C, and hydrolysis time of 90 min. The experimental DH obtained at the optimized condition (63.5%) was close to the predicted DH (64.1%). The enzymatic hydrolysate prepared at the optimal condition showed relatively high amino acid concentration (151.6 ± 1.29 µg/mL) and radical scavenging activity (64.97 ± 0.79%) compared to the boiled sample with values of only 50.1 ± 2.43 µg/mL and 18.36 ± 0.17%, respectively. The resultant hydrolysate had no effect on some of the microorganisms employed in this study. The EBN crumbs hydrolysate inhibited tyrosinase activity with an IC_{50} of 70.22 µg/mL, greater than that of boiled EBN (IC_{50} = 108.9 µg/mL). The results indicated that the EBN crumbs hydrolysate could be further applied in the cosmetic industry as a rich source of nutrients and bioactive compounds for the formulation of beauty products.

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1. Introduction

Edible bird's nest (EBN) is made from the saliva of swiftlet species (*Aerodromus fuciphagus*). It is widely consumed as a health food due to its high beneficial effects on human health and has been considered to be one of the most precious food items in China for thousands of years. The main compositions of EBN are protein (62 - 63%), carbohydrate (25.6 - 27.8%), low amount of lipid (0.14 - 6.28%) and ash (2.1%), (Marcone, 2005). EBN contains 18 amino acids, the most abundant amino acids in the nest are valine, threonine, glutamic acid, aspartic acid, and proline (Ali et al., 2019). It has been used in traditional Chinese medicine as a treatment for malnutrition, a boost to the immune system as well as enhancement to the metabolism and skin complexion (Ma

& Liu, 2012).

In swiftlet farming, EBN crumbs are small fragments obtained from the collecting and processing of EBNs, which are considered as by-products with low economic value. However, several studies have shown that EBN crumbs maintain the important nutrients found in the original material. According to Zainab et al. (2015), EBN by-products contain high levels of protein (47%) and carbohydrates (2.4%). Chan et al. (2015) reported that the free from of N-Acetylneuraminic Acid (NANA) which was found in EBN was proposed to be one of the major compounds responsible for the skin lightening function. Bioactivities of EBN hydrolysates were repoted previously (Ma & Liu, 2012). Enzymatic hydrolysis of proteins released small peptides and free amino acid, leading to an increased nutritional value for food proteins (Khushairay et al., 2014). Many studies mentioned that the enzymatic hydrolysis of EBN results in an increase in amino acids content as well as antioxidant activity (Muhammad et al., 2017; Ali et al., 2019).

In this study, the enzymatic hydrolysis of EBN crumbs was carried out using protease to increase protein solubility and thus maximize its functionality and bioactivity. Response surface methodology (RSM), is employed to optimize the hydrolysis conditions including enzyme loading, hydrolysis temperature, and time (Bezerra et al., 2008). The biological activities including antimicrobial and antioxidant activity, tyrosinase inhibition of the hydrolysate obtained at optimized conditions were examined. In turn, the data will open up new opportunities for applying the EBN hydrolysate in cosmetic industry and other sectors, thus improving the economic value of EBN by-products.

2. Materials and Methods

2.1. Materials

Impurified EBN crumbs were purchased from swiftlets houses in Binh Phuoc province. The raw material was stored in airtight container at room temperature until further analysis. Protease Alcalase® 2.4 L FG (Unit activity 2.4 AU-A/g) used for enzymatic hydrolysis in this study was purchased from Novo Industry (Denmark).

2.2. Sample preparation

The EBN crumbs were prepared according to the method by Amiza et al. (2019a) with some modifications. Prior to hydrolysis, the original EBN crumbs were swollen by soaking in distilled water, then the remaining impurities (dust, feathers, guano,...) were removed manually by the tweezer. Finally, the purified samples were stored at 4 - 8°C until further use.

2.3. Proximate analysis of the raw material

Proximate analysis including crude protein (TCVN 10034:2013), carbonhydrates (AOAC 986.25 mod), ash (EVN-R-RD-2-TP-3496), fat (EVN-R-RD-2-TP-3498), total acid amin (AOAC 994.12) and moisture (EVN-R-RD-2-TP-3496) content of raw purified EBN crumbs. Samples were analyzed and quantified by Eurofins Sac Ky Hai Dang.

2.4. Enzymatic hydrolysis of EBN crumbs

The enzymatic hydrolysis was performed according to the procedure described by Amiza et al. (2019a) with slight modifications.

Purified EBN crumbs were soaked in distilled water at a ratio of 1:100 and then boiled at 100°C for 30 min. After that, the samples were cooled down to room temperature and adjusted to suitable pH and temperature prior to adding protease. The enzymatic hydrolysis was conducted using a magnetic stirrer at specific pH, temperature, time, enzyme loading, and stirring speed. Double-boiled EBN crumbs without any enzyme treatment served as a control experiment in this study. Following the hydrolysis process, the reaction was stopped by inactivating enzyme at 90°C for 10 min in the water bath. The hydrolysate was then centrifuged at 4000 rpm for 10 min. Finally, the supernatant was filtered using filter papers, and the filtrate was stored at 4 - 8°C for further analysis.

2.5. Experimental design

2.5.1. Determination of main factors affecting the enzymatic hydrolysis of EBN crumbs using Plackett Burman Design

In Plackett-Burman design (PB), the independent variables were enzyme loading (X_1: 1%, 5%),

temperature (X_2: 36°C, 60°C), hydrolysis time (X_3: 60 min, 90 min), pH (X_4: 5, 8) and stirring speed (X_5:600 rpm, 1800 rpm), which were employed at two-level (-1 and +1). These variables as well as their ranges were chosen based on the previous published research (Din, 2020). A total of 12 runs were employed as automatically suggested by Design Expert software (Stat-Ease, Inc). Degree of hydrolysis (DH) was set as a response variable. EBN crumbs were hydrolysed under conditions arranged by PB matrix. Data were then analysed by the Design Expert software to determine significant factors for further optimization experiment.

2.5.2. Experimental design for optimization study

In optimization study, a three-level face-centered central composite design (CCD) was used to examine the effect of significant factors on the DH. The independent variables were enzyme concentration (X_1), hydrolysis time (X_2), and temperature (X_3), which were examined at three levels (-1, 0, -1) as shown in Table 1. The DH served as a dependent variable. The ranges of the independent variables were referred from the other previous research (Khushairay et al., 2014; Din, 2020). A total of 20 runs of EBN crumbs enzymatic hydrolysis (including six replicates at the central point) were employed as suggested by Design Expert software (version 11). The EBN crumbs were hydrolysed under conditions arranged by CCD matrix.

2.5.3. Verification of model

To validate the model, three replications of the EBN crumbs hydrolysis were carried out at the predicted optimum condition. The DH of hydrolysate resulted from the enzymatic hydrolysis were determined. One-sample t-test was then performed to compare experimental values and predicted values of DH given by RSM.

2.6. Determination of degree of hydrolysis (DH)

Degree of hydrolysis was determined using trichloroacetic acid (TCA) method according to Morais et al. (2013). The DH was identified by the percentage of soluble protein in TCA 10% in relation to the total protein content of the sample.

Table 1. Experimental design of central composite design

Independent variables	Symbol	Range and levels				
		$-\alpha$	- 1	0	1	$+\alpha$
Enzyme concentration (%)	X_1	2.3	3	4	5	5.7
Hydrolysis time (min)	X_2	50	60	75	90	100
Temperature (°C)	X_3	33.2	40	50	60	66.8

Briefly, 2 mL of hydrolysate aliquot was mixed with 2 mL of TCA 20% solution to obtain the soluble and insoluble fractions in TCA 10%. After 30 min, the mixture was centrifuged at 4000 rpm and the soluble protein content of the supernatant was determined by the Lowry (1951) method and the result was expressed as mg of protein. Bovine Serum Albumin (BSA) was used as the standard. The DH was calculated as follows:

$$\%DH = \frac{Solubleproteincontentin TCA10\%(mg)}{Totalproteincontent(mg)} \times 100$$

2.7. Determination of antioxiant activities

The radical scavenging activity of different EBN crumbs samples was determined using the DPPH assay according to Brand-William et al. (1995) with some modifications.

The EBN crumbs hydrolysates (2 mL) were added to 1 mL of 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, St. Louis, USA) 0.1 mM. The mixture was left for 30 min at room temperature in the darkness. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured using a spectrophotometer at 517 nM. Three mL of DPPH was taken as the negative control and Ascorbic acid at concentration of 5 µg/mL was used as the positive control. The EC_{50} value, the concentration of of different EBN crumbs that could decrease the concentration of DPPH by 50% was then determined. Free radical scavenging activity (RSA) was expressed as inhibition percentage and was calculated using the following formula:

$$\%RSA = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Where:

Abs control: the absorbance of DPPH + Ethanol

Abs sample: the absorbance of DPPH + sample

2.8. Investigation of tyrosinase inhibition ability of EBN hydrolysate

Tyrosinase converts L-tyrosine into an orangered DOPA chrome with a 475 nM absorbance. Samples added to the buffer containing enzymes affect the enzyme activity. The decrease in L-tyrosine metabolism is accompanied by a decrease in optical absorption intensity. The percentage inhibition and IC_{50} values of the samples were calculated from the absorbance measurement at different concentrations.

Investigation of tyrosinase inhibition ability of EBN hydrolysate according to Vanitha & Soundhari (2017) with some modifications. Enzyme tyrosinase from mushroom T3824-25KU (enzyme activity ≥ 1000 unit/mg solid) was provided by Sigma chemical. Pipetting out 100 µL of 100 U/mL tyrosinase into test tubes containing 1.9 mL of the samples and incubate for 30 min at 30°C. Add 1000 µL of 2 mM tyrosine to the mixture, then incubate for 7 min at 30°C. The optical density of the mixture was measured at 475 nM. The positive control is kojic acid. Negative control is prepared by mixing the sample with 100 µL of 0.1 mM phosphate buffer (pH 6.8) without an addition of tyrosinase. Each experiment was carried out in triplicate. An IC_{50} value was used to estimate enzyme inhibition of the sample. The effectiveness of tyrosinase inhibition increases with decreasing IC_{50} values. The percentage of inhibition (I %) was calculated by the following equation:

$$I(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where:

$A_{control}$: OD value of the solution without the test sample.

A_{sample}: OD value of the solution in the presence of the test sample.

The IC_{50} value (µM) is the concentration of a test sample at which the sample can inhibit the activity of the tyrosinase by 50%. This value was calculated based on the equation y = aln (x) + b (y is the percentage inhibition I% and x is the sample concentration) with two values a and b derived from the graph. Substituting y = 50 into the equation we get the value of x.

3. Results and Discussion

3.1. Proximate analysis result

The quantity of protein, lipid and carbohydrate in raw EBN was shown in Table 2. The nutritional value of raw EBN was lower than those found in some regions in Malaysia and Indonesia due to its high moisture content. The highest composition of the EBN collected in Long An, Kien Giang, and Khanh Hoa, was protein (49.4 - 51.17%), followed by carbohydrate (36.93 - 38.53%) (Than et al., 2019). The study performed by Marcone

(2005) revealed that protein was the most abundant EBN component, accounting for 62 - 63%. Besides, the content of carbohydrates and lipids was determined to be 5.62 - 27.26% and 0.14 - 1.28%, respectively.

Table 2. Proximate analysis of raw edible bird's nest[1]

Factor	Unit	Result
Carbohydrates	%	3.42
Total crude protein	%	8.30
Total Fat	%	Not detected (Limit of detection = 0.1)
Ash	%	0.38
Moisture	%	87.9

[1]Results were provided by Eurofins Sac ky Hai Dang.

3.2. Determination of important variables affecting the enzymatic hydrolysis using Plackett-Burman Design

The experiments resulted in a wide range of DH (31.1- 43.3%). The highest DH (43.3%) was obtained at the enzyme loading of 5% at 60°C for 90 min at pH of 5 and rotation speed of 600 rpm. Meanwhile, the lowest DH (31.1%) was obtained at the enzyme concentration of 1% at 36°C for 60 min, pH 5 and at a speed of 600 rpm. ANOVA analysis indicated that three factors including enzyme rate, temperature, and hydrolysis time gave a confidence level greater than 95 % ($P < 0.05$), thus gave a significant impact on the DH (Table 3). In contrast, variables including pH and stirring speed with a confidence level below 95% were considered insignificant. All five factors have a positive effect on DH. Noticeably, DH was most affected by temperature, with an effect value of 4.9, followed by hydrolysis time (4.1) and enzyme loading (3.5).

The PB model suggested that enzyme concentration, temperature and hydrolysis time were three of the most important factors influencing the degree of hydrolysis (DH). As a result, these variables were incorporated in the experimental design of RSM-CCD to evaluate the interaction between these factors as well as determine the optimal condition for the highest DH.

Table 3. ANOVA analysis of Plackett Burman matrix

Factors	Symbols	Levels		Level of influence	
		Low (-1)	High (+1)	Effect	P - value
Enzyme rate (%)	X_1	1	5	3.5	0.0061*
Temperature (°C)	X_2	36	60	4.9	0.0012*
Hydrolysis time (min)	X_3	30	90	4.1	0.0029*
pH	X_4	5	8	0.6	0.5283
Stirring speed (rpm)	X_5	600	1800	0.6	0.3456

*: significant at $P < 0.05$

3.3. Optimization of hydrolysis conditions for maximum DH using the RSM-CCD model

3.3.1. Experimenal data and ANOVA for degree of hydrolysis

Table 4 showed that DH of EBN crumbs hydrolysate ranged from 42.9 to 63.9%. The highest DH (63.9%) was obtained at enzyme loading of 4% for 100 min at temperature of 50°C. Table 5 showed the ANOVA of two-factor interaction (2FI) as suggested by Design Expert software. At a 95 % confidence level, the model was considered significant with a P-value < 0.001. A P-value of 0.0859 (> 0.05) indicates that the lack of fit is not significant. This non-significant lack of fit is favourable in an optimization study, as it illustrates the model's ability to fit well with experimental data. Besides, the calculated regression coefficient R^2 was 0.9567. The predicted R^2 (0.7228) corresponds to the adjusted R^2 (0.9177) with less than 0.2 difference indicating that the model is adequate. Adeq precision (14.664) is greater than 4 is desirable that the model is suitable for practical use. Additionally, the coefficient of variation (% CV) also indicates the accuracy of the experiment, those with low reliability often have a high % CV. In this model, a % CV value of 3.36 indicates that the experiments performed are reliable.

From the ANOVA, it was found that all linear model terms (X_1, X_2 & X_3) with a P-value < 0.05 affected the DH significantly. Quadratic values include X12, X22 and interaction terms X_1X_3 and X_2X_3 also showed a high level of significane. In contrast, X_2^2 and X_1X_2 were insignificant terms at $P = 0.05$. The model reduction was carried out to simplify the equation by excluding insignificant terms.

The final equation in terms of coded factors given by Design Expert software was:

$$Y = 61.5 + 1.22X_1 + 2.76X_2 + 1.81X_3 + 1.93X_1X_3 \breve{} 1.9X_2X_3 \breve{} 5.02X_1^2 \breve{} 3.81X_3^2$$

Where:

Y: degree of hydrolysis (% DH);

X_1, X_2, X_3 are enzyme loading (%), hydrolysis time (min), and temperature (°C), respectively.

The quadratic regression equation implies that enzyme loading, hydrolysis time and temperature have significant impacts on the enzymatic hydrolysis of EBN crumbs using protease enzyme. These variables showed positive coefficients, indicating that each factor contributed to an increased DH. According to the equation, the DH is most affected by hydrolysis time (2.76), followed by temperature (1.81) and enzyme loading (1.22). Additionally, the interaction between enzyme concentration-temperature (X_1X_3) and hydrolysis time-temperature (X_2X_3) also contribute to the improvement of hydrolysis efficiency.

3.3.2. Response surface plots for interaction effect

Figure 1(a) illustrated the impact of the interaction between enzyme concentration (X_1) and temperature (X_3) on DH. The maximum of DH is obtained at intermediate enzyme loading and temperature while hydrolysis time reached 90 min. On the other hand, lower or higher than the intermediate level of these two factors result in decreased DH. It's assumed that more active sites are available in the enzyme at higher enzyme concentration, thus resulting in great cleavage of the peptide bonds and provided higher DH. However, when increasing the enzyme loading to over 5%, the DH no longer increased because the enzyme might be saturated with the substrate, (Mackei, 1982). For most enzymatic hydrolysis reactions, the rate of hydrolysis increased when the temperature increases. In this study, the lower DH of 40 to 50 may be due to the insufficient energy provided for the protease to bind with the substrate. However, DH was not significantly affected by temperature from 50°C - 60°C, this is in agreement with the research carried out by Silva et al. (2010) using the Protamex.

Figure 1(b) showed that the DH increases at intermediate enzyme loading throughout the hydrolysis time used in this study. An increase in hydrolysis time allowed the enzyme to act more extensively on the protein, thereby increasing DH. This is in agreement with Amiza et al. (2019b) and Ovissipour et al. (2010). However, Khushairay et al. (2014) and Nurfatin et al. (2016) reported that, after an initial increase in DH, a decrease in the rate of hydrolysis of EBN was observed when incubated for more than 1 - 1.5 h and the conversion was entering a stationary phase. Despite having more cleavage sites, the extent of hydrolysis of EBN depends on the cleavage specificity of the enzyme and the accessibility of peptide bonds to each enzyme. Besides,

Table 4. DH values according to Central composite design-Response surface methodology

Run	Factors			Degree of hydrolysis (% DH)	
	X_1	X_2	X_3	Experimental	Predicted
1	3	90	40	58.9	56.9
2	4	75	50	62.6	62.0
3	4	75	50	62.3	62.0
4	4	75	50	63.1	62.0
5	5	90	60	58.1	57.6
6	5	90	40	53.8	53.9
7	4	75	50	62.6	62.0
8	3	60	40	47.3	46.0
9	3	90	60	53.2	52.6
10	4	50	50	54.1	55.7
11	5	60	60	56.8	57.2
12	5.7	75	50	49.8	49.7
13	4	75	33	46.1	48.0
14	5	60	40	47.2	45.9
15	2.3	75	50	42.9	45.6
16	4	100	50	63.9	64.9
17	4	75	67	53.4	54.1
18	4	75	50	62.1	62.0
19	3	60	60	51.5	49.6
20	4	75	50	59.7	62.0

Table 5. ANOVA of Central composite design-Response surface methodology

Source	Sum of Squares	Mean square	F-value	*P*-value	
Model	768.09	85.34	24.54	< 0.0001*	significant
X_1 – Enzyme rate	20.19	20.19	5.81	0.0367*	
X_2 – Time of hydrolysis	103.97	103.97	29.90	0.0003*	
X_3 – Temperature	44.59	44.59	12.82	0.0050*	
X_1X	3.65	3.65	1.05	0.3300	
X_1X_3	29.65	29.65	8.53	0.0153*	
X_2X_3	28.88	28.88	8.31	0.0163*	
X_1^2	371.29	371.29	106.78	< 0.0001*	
X_2^2	5.25	5.25	1.51	0.2475	
X_3^2	216.25	216.25	62.19	< 0.0001*	
Lack of fit	27.48	5.5	3.77	0.0859	not significant

$R^2 = 0.9567$; C.V. % = 3.36; adjusted $R^2 = 0.9177$; predicted $R^2 = 0.7228$.
*: significant at = 0.05.

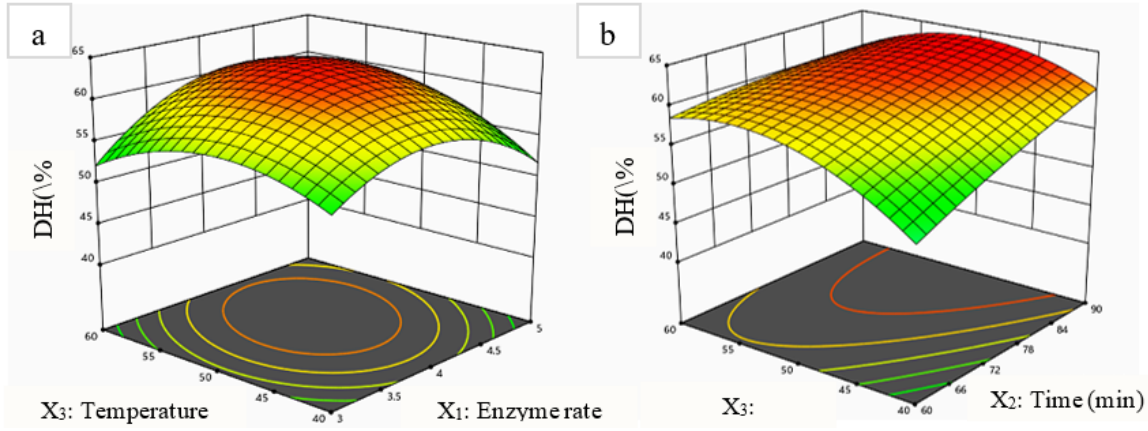

Figure 1. Response surface plot for the interaction between two factors: *(a) enzyme loading (X_1), temperature (X_3) when hydrolysis time was conducted for 90 min; hydrolysis time (X_2) and temperature (X_3) when enzyme loading was fixed at* 4%.

Arihara (2006) stated that, 100% solubility will never achieve which is related to peptide-peptide interactions and partly to the presence of glycoprotein that can not be hydrolyzed. The results obtained from Figures 1(a) & (b) indicated that an increase in DH can be achieved by increasing enzyme concentration, hydrolysis time, and temperature up to certain levels.

3.3.3. Optimization of degree of hydrolysis and verification of the optimal condition

The optimal hydrolysis conditions of EBN crumbs for the highest DH were predicted at 4% of enzyme loading, 51°C of temperature for 90 min. The maximum DH value under this optimal condition was 64.1% .

The maximum DH in this study is higher than that of enzymatic hydrolysis of EBN using alcalase (37.92%), and protamex (33.88%) (Amiza et al., 2019b). However, it is relatively lower compared to that of EBN hydrolysis using Pancretin 4NF (86.5%) and alcalase (82.7%) within hydrolysis time from 1 - 1.5 h. The difference in DH between these studies could be attributed to the difference in source of material used. The major nutrient components of EBN are carbohydrates and glycoproteins. Hydrolysis of glycoprotein often results in a wide range of molecular weight peptides, which are difficult to separate and characterize. According to Muhammad et al. (2015), approximately 40% of the hydroxyl amino acids in EBN are in the position of carbohydrate-peptides linkages, thus making the EBN difficult to hydrolyze thoroughly.

The DH is also depended on the type of enzymes as well as the different range of parameters applied during enzymatic hydrolysis. Each type of enzyme will work differently during proteolysis depending on their preferred amino acid to be cleaved during the hydrolysis process.

To validate the model, three replicates of EBN crumbs hydrolysis were performed under the optimal conditions suggested by the software. According to one-sample t-test, there was no significant difference between the experimental value (63.5% ± 0.76) and the predicted value (64.1%). This means that the equation fits well with the experimental data in this study.

3.4. Quantitative analysis of amino acids

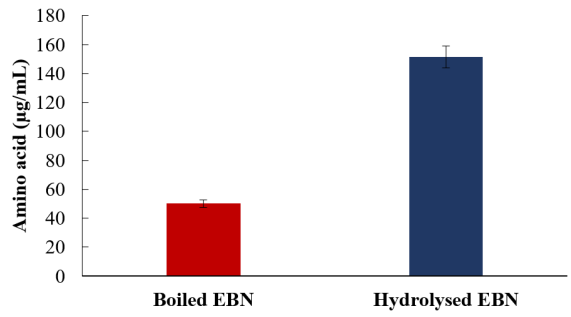

Figure 2. Amino acid concentration in the hydrolyse. EBN: edible bird's nest.

According to Figure 2, hydrolysed EBN crumbs have a total amino acid concentration of 151.6 ± 1.29 µg/mL, whereas boiled EBN crumbs have a concentration of 50.1 ± 2.43 µg/mL. After enzymatic hydrolysis, the total amount of free amino acids in the EBN crumbs is three times higher than that in the doubled-boiled samples. This could be due to double-boiled EBN does not undergo enzymatic hydrolysis and the boiling temperature was not able to break the strong peptide bonds.

The result demonstrated the efficiency of the enzymatic hydrolysis in cleaving peptide bonds to release soluble protein and free amino acids.

3.5. DPPH radical scavenging assay

The EBN hydrolysate exhibited higher DPPH radical scavenging activity 64.97 ± 0.79% than that of double-boiled EBN 18.36 ± 0.17% (Figure 3). As compared with the double-boiled preparations, the EBN prepared with protease may have more active amino acids or peptides, which could scavenge free radicals.

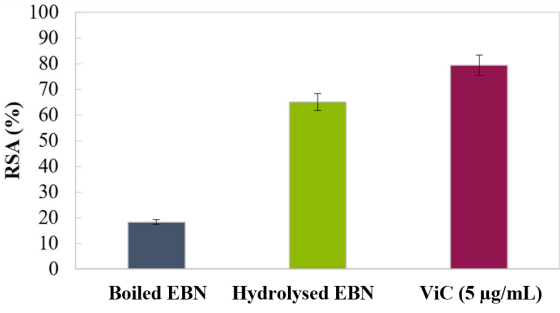

Figure 3. Free radical scavenging activity (RSA) of the hydrolysate. EBN: edible bird's nest.

The EBN crumbs hydrolysate in this study had greater DPPH scavenging activity compared to the hydrolysis process employed by alcalase (RSA of 44.8%) and papain (RSA of 49.78%) (Muhammad et al., 2015). However, it is lower compared to the EBN hydrolysis by Bromelain with antioxidative activity of 82.55 ± 0.73% (Bui, 2020). Previous research also proved that the antioxidative activity of protein hydrolysates depends on the kind of protease enzyme and hydrolysis conditions employed (Jun et al., 2004). During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on en-

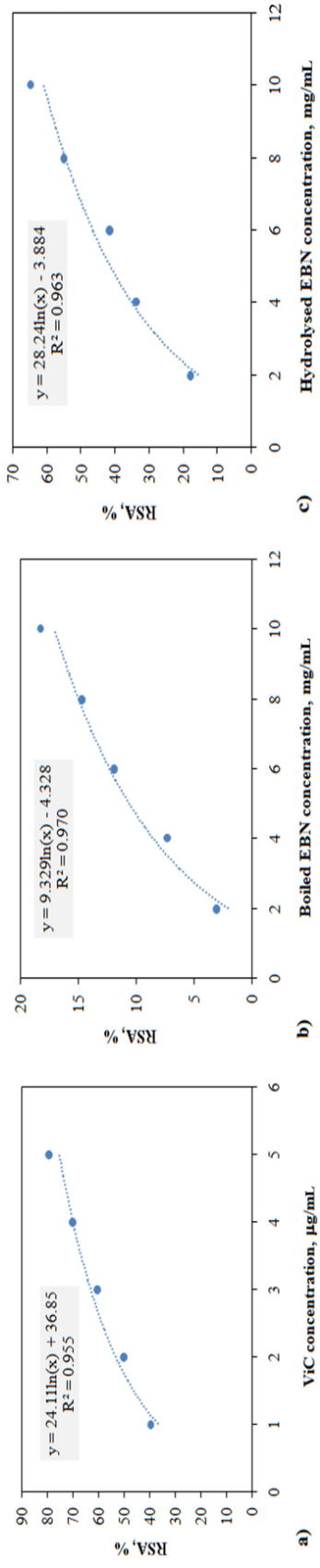

Figure 4. Antioxidant activity of vitamin C and different edible bird's nest (EBN) crumbs hydrolysate (a) Vitamin C, (b) Boiled EBN, (c) Hydrolysed EBN. RSA: radical scavenging activity.

zyme specificity. Changes in size, level, and composition of free amino acids and small peptides significantly affect the antioxidative activity (Wu et al., 2003).

In fact, Figure 4 shows that antioxidant capacity of the EBN crums hydrolysate was higher than the boiled ENB through IC_{50} value. The results indicated that bioactive substances produced by enzymatic hydrolysis of EBN crumbs could be further applied in the cosmetic industry as a rich nutrient for the formulation of beauty products, enhancing both their economic and nutritional value.

3.6. Tyrosinase inhibition of EBN crumbs hydrolysate

According to the results of the tyrosinase enzyme inhibitory activity, kojic acid was significantly inhibited with an IC_{50} of 19.69 µg/mL, boiled EBN had a weak inhibitory activity with an IC_{50} value of 108.9 µg/mL, and EBN crumbs hydrolysate prepared under optimal conditions gave a moderate inhibitory activity with an IC_{50} value of 70.22 µg/mL (Figure 5).

Tyrosinase inhibition of the EBN crumbs hydrolysate (Figure 6) was about 56%, which was comparable to bird's nest extract in Khanh Hoa with 60% of enzyme inhibitory (Le et al., 2017). The results demonstrated a potential use of ENB crumbs hydrolysate in skin care products such as foam, lotion or serum.

4. Conclusions

Optimal conditions for the enzymatic hydrolysis of EBN crumbs were found at an enzyme loading of 4%, temperature of 51°C, and hydrolysis time of 90 min. The experimental DH achieved at the optimized condition (63.5%) was close to the predicted DH (64.1%) suggested by the model. The enzymatic hydrolysate prepared at the optimize condition showed relatively high amino acid concentration (151.6 ± 1.29 µg/mL) and DPPH radical scavenging activity (64.97 ± 0.79%) compared to those of the boiled sample with only 50.1 ± 2.43 µg/mL and 18.36 ± 0.17%, respectively. In addition, the EBN crumbs hydrolysate showed to inhibit tyrosinase at an IC_{50} of 70.22 µg/mL, which demonstrated potential applications in skin care and beauty products.

Figure 5. Tyrosinase inhibition reaction at concentrations ranging from 0 to 100 (µg/mL). (a) Acid kojic; (b) Boiled EBN; (c) Hydrolysed EBN. EBN: edible bird's nest

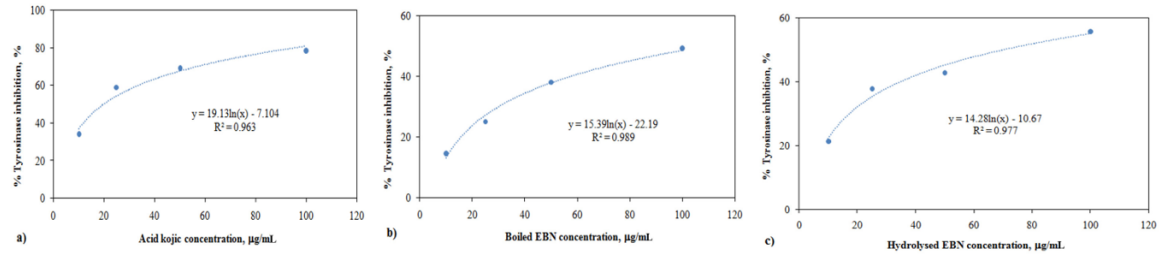

Figure 6. Correlation between substance concentration and tyrosinase inhibitory activity. (a) Kojic Acid, (b) Boiled EBN, (c) Hydrolysed EBN. EBN: edible bird's nest.

Conflict of interest

The authors have no conflicts of interest to declare.

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Isolation and characteristics of *Pseudomonas fluorescens* to inhibit *Phytophthora palmivora* causing rot disease in durian

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ABSTRACT

Plant growth promoting Rhizobacteria (PGPR) have long been selected as biocontrol agents for plants. The PGPR are beneficial bacteria that live in plant roots and enhance plant growth by various of mechanisms. The PGPR have many species of the bacterial genus Pseudomonas, in which the *Pseudomonas fluorescens* strains is suitable for application as a biocontrol agent due to its abundance in natural soil and plant root systems. *Pseudomonas fluorescens* strains have important characteristics such as the ability to adhere to soil particles and to rhizomes, the ability to synthesize antibiotics, and to produce hydrolytic enzymes. Furthermore, *P. fluorescens* strains also possess plant growth promoting characteristics such as proteolysis, phosphate solubilization, iron chelation, and phytohormone production. Durian is a crop that has been expanded in Southeast Asian countries but the rate of durians infected with root, stem and fruit rot caused by *P. palmivora* is quite large. In this study, five native *P. fluorescens* isolates were isolated from 70 soil and rhizome samples collected from 10 durian growing provinces and evaluated for their ability to antagonize *P. palmivora* in petri plates. These isolates were gram negative, small, single isolated rods without sporulation when observed under microscope. All of them tested positive for catalase test, oxidase test, starch hydorolysis, gelatine liquefaction, H_2S, citrate utilization and negative with indole, Voges-proskauers, methyl red. All of 5 native *P. fluorescens* isolates were capable of phosphate solubilizing activity, N_2 fixation, siderophore and IAA production. The study showed that *P. fluorescens* P. fDN strain was able to inhibit *P. palmivora* causing rot disease in durian with 51.85% inhibition of radial growth.

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1. Introduction

Pseudomonas sp. are considered biological control agents because they are widely present in agricultural soils and have many suitable characteristics in the PGPR group. Around the world, many studies have been established to exploit the potential of pseudoluminescent bacteria, in which the most concentrated aspects are phosphate solubilizing activity, N_2 fixation, siderophore and IAA production. Reported by Glick et al. (1999) showed that IAA-producing rhizobacteria enhanced root length, which is one of the rhizobacteria promoting plant growth activity. Gupta et al. (2002) isolated fluorescent *Pseudomonas* strains capable of producing IAA from potato rhizosphere. Report by Pandey et al. (2013) also showed that, *Pseudomonas* strains are indeed rhizosphere endophytes promoting good growth of sunflower (*Helianthus annus*). In Vietnam, Tran et al. (2010) studied the effect of *P. fluorescens SS01* on black mold wilt (*Aspergillus niger*) on peanuts, showed that this strain has the ability to survive 30 days after inoculation with high

density and decrease in density at later times. Ngo et al. (2011) determined the level of nitrogen fertilizer replacement of *Pseudomonas* sp. BT1 and BT2 with high yielding rice plants grown in pots. In the case of replacing 50% N with *Pseudomonas*, the rice yield in potting could be increased by 23.9% compared to the control. Chu et al. (2020) isolated and selected *Pseudomonas* three strains capable of stimulating the growth of peanut under saline conditions both in vitro and in nursery. *P. fluorescens* had been reported to manage several diseases caused by soil borne pathogens (Hoffland et al., 1996; Wei et al., 1996). The purpose of this study is to isolate and characterrize *P. fluorescens* antagonists against rot durian disease caused by *Phytophthora palmivora*.

2. Materials and Methods

2.1. Isolation and morphological description

2.1.1. Soil sample collection

Samples were collected from the rhizosphere soils of durian farms at Tay Ninh, Binh Phuoc, Binh Duong, Dong Nai, Daklak, Dac Nong, Lam Dong, Vinh Long, Ben Tre, Dong Thap provinces. Samples were taken as follows: first, the topsoil of about 10 cm was removed and a metal spoon was used to collect soil at a depth of 10 - 20 cm. Then, 2 - 3 tablespoons of soil was taken at each point and the soil samples around the roots of many points were pooled to have a soil sample of 500 g. Thus, fives samples taken from a garden (or farm) included 4 diagonal points and 1 center point of the gardens. Seventy soil samples were collected from the rhizosphere of durian farms. The soil intimately adhering to the roots was collected and mixed to provide a composite soil sample.

2.1.2. Isolation of *P. fluorescens* isolates

Methods of Vlassak et al. (1992) was used to isolate *P. fluorescens*. Ten g of soil sample was put into a 90 mL conical flask of salt water and shaken for 15 min on a shaker. Then, it was aspirated and serially diluted the suspension to concentrations from 10^{-2} to 10^{-4}. About 0.1 mL of the corresponding diluent was added onto a sterilized petri dish containing King's B medium (KB). The petri dishes were incubated at room temperature (30°C ± 2°C) for 24 - 72 h. Each dilution was repeated 3 times. The growth of *Pseudomonas* colonies on the KB was observed during 24 - 72 h.

2.1.3. Morphological characterization

Pseudomonas strains were purified and studied for colony morphology and pigmentation by gram staining, described on specialized media. Cultural characterization of isolates was observed by different colony characteristics such as shape, size, surface, margin, color, odor, pigment, etc., and was recorded based on Bergey's Handbook of Definitive Bacteria (Holt et al., 1994). Cell shape and gram response were also recorded using the standard procedure provided by Barthalomew & Mittewar (1950).

2.2. Biochemical and Physiological Characterization

2.2.1. Starch hydrolysis

Ten mL the overnight culture of the isolates was added to the center of a sterile starch agar plate and incubated at 30 ± 2°C for 24 - 48 h. After incubation, the plates were submerged in an iodine solution. A transparent area around the colony was considered a positive reaction for the test.

2.2.2. Hydrogen sulfide test

A culture stick was used to take the bacterial solution of overnight cultures of the isolates. Then, it was pierced deeply into sterilized hydrogen Sulfide Indole Motility agar and incubated for 48 h at 30 ± 2°C. Observation visualization of black colour along the line of inoculation indicated a positive reaction for the test.

2.2.3. Indole production

The cultured bacteria were inoculated overnight into sterilized SIM agar plates and incubated for 48 h at 30 ± 2°C. After incubation, 10 drops of Kovac's indole reagent was added to each tube. The observed red production was noted as positive for indole production.

2.2.4. Catalase test

This test was performed to study the presence of catalase enzyme in bacterial colonies. Fresh cultures of purified isolates were taken on a slide

and a drop of H_2O_2 (30%) was added. The appearance of gas bubbles indicated the presence of the catalase enzyme.

2.2.5. Oxidase test

The isolates were inoculated on a petri dish of sterile trypicase soy agar and incubated for 24 h at 30 ± 2°C. After incubation, 2 - 3 drops of N, N, N', N' - tetramethyl - p -phenylenediamine dihydrochloride (Wurster reagent) were added to the growth surface of each test organism. Isolations reported a color change to chestnut were as oxidase - positive.

2.2.6. Carbohydrate utilization

Purified bacteria were inoculated into peptone broth containing different carbohydrates (lactose, sucrose, dextrose and mannitol) to assess carbohydrate fermentation. The change in colour of peptone broth was observed for utilization of particular carbohydrate present in broth.

2.2.7. Methyl red test

Bacteria were inoculated into test tubes containing sterilized glucose phosphate broth and incubated at 28 ± 2°C for 48 h. After that, 5 drops of methyl red indicator were added to each tube and shaken gently. If the test tube was red, it was recorded as positive and yellow as a negative reaction. Methyl red test helped to distinguish the concentration of H^+ present in the medium after fermentation by microorganisms.

2.2.8. Voges Prausker's test

The test sample was inoculated into sterilized glucose phosphate broth and incubated at 37°C for 48 h. After incubation, 10 drops of Baritt reagent A were added and shaken gently, then adding 10 drops of Baritt reagent B. If the test tubes was pink, it was recorded as positive for the test.

2.2.9. Gelatin liquefaction

The cultured bacterial samples were inoculated overnight into sterilized nutrient gelatin deep test tubes and incubated for 24 h at 30 ± 2°C. Then, the tubes were kept in a refrigerator for 30 min at 4°C. The isolates showing liquefied gelatin were taken as positive and which resulted in solidification of gelatin on refrigeration were recorded as negative for the test.

2.2.10. String test

A sterile toothpick was used to collect the bacterial biomass grown on the medium plate and spread it on a drop of 3% KOH solution on a clean slide (the more biomass, the clearer was observed). The spinning ability of the bacteria was recorded.

2.2.11. Pigment pyoverdine test

The test colonies were inoculated on King's B agar and incubated at 25°C for at least 2 days. The plate was observed under UV light, *P. fluorescens* secretes a yellow green pigment pyoverdine that diffused in the medium along the inoculum and fluoresced under UV light.

2.2.12. Pigment pyocyanin test

Bacterial strains were inoculated on King's A medium, incubated at 30°C for 48 - 72 h. *Pseudomonas fluorescens* did not appear blue pyocyanin pigment on King's A medium. This test helped to distinguish between *P. fluorescens* and *P. aeruginosa* strains.

2.3. Investigation of some biological activities

2.3.1. IAA (β - Indol Acetic Acid) produce test

A color reaction with the denatured Salkowski reagent was used for determination. The IAA content was produced by *P. fluoresces*. Bacteria were shaken in TSB medium supplemented with 0.1 g/L tryptophan in the dark. After 4 days of shaking at 150 rpm, 30 ± 2°C, 1 mL of inoculum was collected and centrifuged to remove biomass, then 2 mL Salkowski modified reagent was added. Tthe mixture was incubated for 1 h in the dark, a positive reaction gave a light pink to red color.

2.3.2. Nitrogen fixtation test

Bacteria were grown in a nitrogen-free medium (MNFM) complemented by the addition of bromophenol blue as a pH indicator. Bacterial strains that changed the color of the medium after 2 days of culture were recorded (Gothwal et al., 2007; Latt et al., 2018).

2.3.3. Phosphate solubilization test

According to the method of Nautiyal (1999), the bacterial biomass was dissolved in $Ca_3(PO_4)_2$ phosphate buffer (0.5%) and diluted to a density of about 10^8 CFU/mL. About 0.002 mL of bacterial biomass was taken in physiological saline and inoculated at 1 point on Pikovskay agar, incubated at 30°C. The appearance of lysis rings around the colony was observed.

2.3.4. Dual culture test to evaluate the ability to inhibit *Phytophthora palmivora*

textitPhytophthora palmivora was isolated from infected durian fruit in Cam My district, Dong Nai province. It was isolated on V3 juice agar and then maintained at room temperature. For this experiment, a 7 days old V3 plate of *P. palmivora* was used.

Control plates comprised only PDA with *P. palmivora* and sterilized water. Three plates were prepared for each observation and incubated at room temperature (± 28°C) for 5 - 7 days. Inoculation of pathogen was done by placing a 0.5 mm diameter plug of *P. palmivora* facing down, 1.5 cm from the centre of the PDA petri dish. *P. fluorescens* strains were grown in nutrient broth for two days before. A filter paper was dipped in solution of *P. fluorescens* and thawed on sterile filter paper. It was then placed 3 cm away from the *P. palmivora* isolate. Growth of *P. palmivora* was taken by measuring the diameter of the mycelium growth. Data of the percentage growth inhibition (PGI) was calculated using the formula of Zivkovic et al. (2010).

$$PGI(\%) = \frac{R_1 - R_2}{R_1} \times 100$$

Where:

R1: Growth of pathogen alone without antagonist (control)

R2: Growth of pathogen along with the antagonist

2.3.5. Data prpcessing method

Data were collected, synthesized, calculated using Microsoft Excel, analyzed by 1-factor ANOVA and graded test using MINITAB 16.

3. Results and Discussion

3.1. Isolation of *Pseudomonas fluorescens*

Isolation results obtained 16 samples showing *Pseudomonas* sp. (16/56 bacteria isolated) from 70 samples of durian root soil collected in 10 durian growing provinces including Binh Duong, Binh Phuoc, Tay Ninh, Tien Giang, Vinh Long, Ben Tre, Dong Nai, Lam Dong, Dak Lak and Dak Nong. The 16 samples belonging to the genus *Pseudomonas* were identified based on the characteristics of gram negative, rod shaped, not fermenting sugars, clear colonies, regular or irregular borders, and mucilage.

However, resulting from the string test with 3% KOH, purifying and observing the ability to fluoresce under UV light of the bacterial strains showed that only 5 samples were able to fluoresce suspected *P. fluorescens*. Five samples were collected from Binh Duong, Dong Nai, Dong Thap, Tien giang, Lam Dong.

3.2. Cultural and morphological characterization

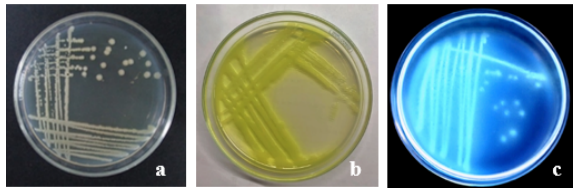

Figure 1. (a) *Pseudomonas fluorescens (P. F_{DN},* colonies on King's B agar medium, (b) *P. Fluorescens* secretes yellow-green pigment pyoverdine on King's B, (c) Colonies of *P. Fluorescens* was fluoresced under UV light on King's B.

Five *P. fluorescens* isolates, based on their colony morphology on King's B agar medium, cell morphology and gram reaction (Figure 1). All the isolates developed small to medium, smooth, glistening colonies, yellowish green colour with light green pigmentation. These isolates were gram negative, small, single isolated rods without sporulation when observed under microscope. This finding was similar to the description of the morphological characteristics of *P. fluorescens* P60 in the study of Soesanto et al. (2011).

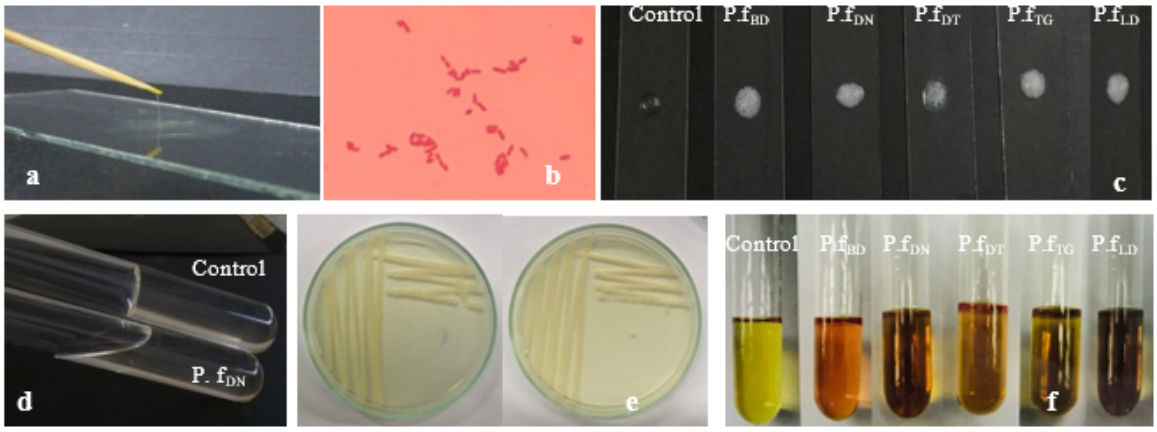

Figure 2. (a) String test spinning ability of *Pseudomonas* with KOH 3%, (b) Microscopic observation of *Pseudomonas*, (c) Catalase test of 5 *P. fluorescens* isolates, (d) Gelatine test, (e): Pyocyanin production test, (f) IAA production test with the modified Salkowski reagent.

Table 1. Biochemical and physiological characteristics of *P. fluorescens* isolates

Isolates	Oxidase test	Catalase test	Starch hydrolysis	Gelatine liquefaction	H_2S	Indole	MR	VP	Citrate utilization
P. f_{BD}	+	+	+	+	+	-	-	-	+
P. f_{TG}	+	+	+	+	+	-	-	-	+
P. f_{DT}	+	+	+	+	-	-	-	-	+
P. f_{DN}	+	+	+	+	+	-	-	-	+
P. f_{LD}	+	+	+	+	+	-	-	-	+

MR -Methyl red; VP – Voges Praskaur's test; + Positive result; - Negative result.

Table 2. Biological characteristics of *P. fluorescens* isolates

Isolates	Nitrogen fixtation test	Pyocyanin production	IAA production	Siderophore production	Phosphate solubilization
P. f_{BD}	+	-	+	+	+
P. f_{TG}	+	-	+	+	+
P. f_{DT}	+	-	+	+	+
P. f_{DN}	+	-	+	+	+
P. f_{LD}	+	-	+	+	+

+ Positive result; - Negative result.

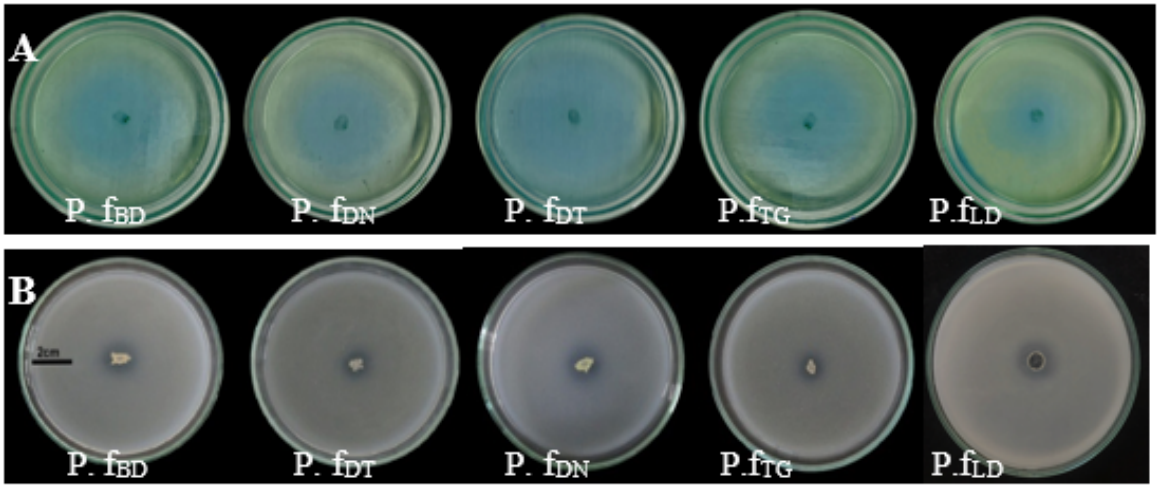

Figure 3. (A) Nitrogen fixtation test of *P. fluorescens* on a nitrogen-free medium after 2 days, (B) Phosphate solubilize test on Pikovskaya medium after 7 days.

3.3. Biochemical and physiological characterization

After the study of cell morphology, the isolates of the *P. Fluorescens* (5 isolates) were tested for different biochemical such as IMVIC test, oxidase test, catalase test, carbohydrate fermentation, denitrification, H2S production, starch hydrolysis, gelatin liquefaction etc. (Table 1; Figure 2). All 5 isolates from *P. fluorescens* showed positive results from catalase test, oxidase test, starch hydrolysis, gelatine liquefaction, H2S and citrate utilization whereas which was negative for Voges Prausker's, methyl red and indole test. In this study, 5 *P. fluorescens* isolates had diffused pyoverdin pigment capable of fluorescence into King's B medium, that showing the potential of using pigments of these *P. fluorescens* to create iron biosensors (Meyer, 2000) for future research and applications. The biological characteristics studied in Table 2 showed that the 5 *P. fluorescens* isolates were capable of solubilizing phosphate, fixing N2, producing siderophore and IAA. Many studies has shown that strains of *Pseudomonas* spp. were used in biocontrol because they produced antibiotic with antifungal abilities in vitro (Raaijmakers et al., 2002). The results obtained suggest the potential to use native *P. fluorescens* samples of further studies.

3.4. Dual culture test to evaluate the ability to inhibit *Phytophthora palmivora*

Antagonistic potential of 5 *Pseudomonas fluorescens* isolates (P. F_{BD}, P. f_{TG}, P. f_{DT}, P. f_{DN}, P. f_{LD}), were tested against *P. palmivora* in dual culture under in vitro conditions (Figure 3). The growth of the fungus was lesser as compared to the control plate. Among them P. f_{DN} performed best which gave 51.85% inhibition of radial growth followed by P. f_{LD} (43.71%), P. f_{TG} (42.96%) to *P. palmivora*, whereas, P. f_{DT} strain was not inhibition of radial growth (0%), (Table 3). The results in the Table 3 showed that there was no significant difference among the strains in controlling the growth inhibition of *P. palmivora*. Gade & Lad (2019) recorded that *P. fluorescens* was able to maximally inhibit the growth of the mycelium *Phytophthora parasitica* by 34.35%. *Pseudomonas fluorescens* strain is collected in different geographical areas, it is possible to produce different secondary metabolites, so the difference in percentage inhibition of different mycelium for the pathogen will test (Meena et al., 2012; Singh et al., 2013). Research of Laha et al. (1992) showed that *P. fluorescens* resistance to *Rhizoctonia batticola* in agar plate might be due to the production of siderophores or possibly to volatile antifungal compounds.

Table 3. Efficacy of *Pseudomonas fluorescens* against *Phytophthora palmivora*

Isolates	Diameter mycelial growth (mm)	Percentage growth inhibition (%)
Control	90	N/A
P. f_{BD}	71.33	$40.30^{c} \pm 0.30$
P. f_{TG}	70.67	$42.96^{b} \pm 0.74$
P. f_{DT}	90	$0.00^{d} \pm 0.00$
P. f_{DN}	66.67	$51.85^{a} \pm 0.74$
P. f_{LD}	70.33	$43.70^{b} \pm 0.74$

Treatments with the same letters did not differ significantly ($P \leq 0.05$) according to the Duncan's multiple range test.

4. Conclusions

Seventy soil samples were collected from rhizospheric soil at durian farms in 10 provices in Vietnam. Five *Pseudomonas fluorescens* isolates were isolated and evaluated in detail for their morphological and biochemical characteristics. All the isolates of *Pseudomonas fluorescens* in this study showed small to medium, smooth, glistening colonies, yellowish green colour with light green pigment on King's B medium. These isolates were small, single, gram negative isolated rods without sporulation when observed under microscope. All of these were positive with catalase test, oxidase test, starch hydorolysis, gelatine liquefaction, H_2S, citrate utilization and negative with indole, Voges-proskauers, methyl red. They were also able to produced siderophore and diffused pyoverdin pigment of fluorescence into King's B medium. Study showed that, all 5 *P. fluorescens* isolates were capable of phosphate solubilizing activity, N_2 fixation, siderophore and IAA production, inhibition of *Phytophthora palmivora*. These product from *Pseudomonas fluorescens* isolates have multiple roles in exerting the growth of plant. Therefore, these isolates can be utilized for biofertilizer formulation under local agroclimatic conditions.

Among five *Pseudomonas fluorescens* isolates, P. f_{DN} strains has against best with *Phytophthora palmivora* which gave 51.85% inhibition of radial growth. Future study with these isolates using them in pot cultures and followed by field experiments will help in establishing their potential to be used as biofertilizers. The data obtained in the present study suggest that five *Pseudomonas flourescence* isolates, especially, P. f_{DN} would be ideal organisms for further study in pot culture and field experiments to exploit their PGPR potential for a good biofertilizers production.

Conflict of interest

The authors have no conflicts of interest to declare.

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Water desalination of *Chlorella vulgaris*

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ABSTRACT

Saltwater intrusion is a common phenomenon in Southern Vietnam, but salinization is becoming more serious due to the rising sea level related to climate change. Among potential methods for water desalination, the application of halophytic microalgae is gaining high interest. This study investigated the effect of *Chlorella vulgaris* (*C. vulgaris*) microalgae on reducing water salinity at different media (Bold Basal Medium and Sea Salt Medium) and in different salt concentrations (1 - 30 g/L). The results indicated that *C. vulgaris* microalgae had good growth in all mediums used and contributed to lowering the salt content from 20% to 40% after 15 days of cultivation.

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1. Introduction

Saltwater intrusion is a popular phenomenon in the Mekong River Delta, one of the most productive agricultural areas in the world. However, when the sea level rises relating to climate change, the salinization of this area is becoming more serious. During the last 5 years, saltwater has reached more than 50 km from the coast of Vietnam and threatened the agriculture of provinces along the Mekong River Delta. In general, there are not many options for desalinating water in this area. The popular method for farmers is building channels and reservoirs but this solution is not sustainable because salt will be accumulated on the land, resulting in another contamination phenomenon. Another solution is applying reverse osmosis filtration systems which consume high energy and require a high cost for membrane replacement. In considering sustainable water treatment solutions, the application of microalgae is always a potential solution (Abdel-Raouf et al., 2012). Earlier studies have reported that some microalgae not only are useful in removing a wide range of substances pollution from industrial and agricultural wastewater but also contribute to lowering salt content in water depending on its salt tolerance (Delrue et al., 2016; Gan et al., 2016).

Salt tolerance is the ability of plants to grow and complete their life cycle on substrates containing high concentrations of soluble salts (Wang et al., 2013). Survival rates were used for uncultivated and long-lived species to determine

salt tolerance (Cheeseman, 1988). The degree of growth reduction and tolerance to stress conditions varied among different plant species. In general, plants can be classified into glycophytes and halophytes. Halophytes, also known as salt-loving plants, are plants that are tolerant to the effects of salinity and possess salt-responsive genes and proteins to counteract the adverse effects of salinity (Askari et al., 2006). Meanwhile, glycophytes, also known as salinity-sensitive plants, cannot tolerate high salinity. The salinity tolerance degree of halophytes also varies between species (Yensen et al., 2016). Less tolerant halophytes reduce their growth in saline environments in response to salinity while showing better growth in non-saline soils (Zhu, 2001). Some recent studies have shown that some popular halophytic microalgae strains such as *Chlorella, Chlorococcum, Desmodesmus, Scenedesmus* and *Monoraphidium* could survive and grow in water containing high salt concentrations and remove approximately 30% chloride in water (Figler et al., 2019; Sahle-Demessie et al., 2019; Barahoei et al., 2021). However, the salt tolerance as well as water desalination effect of microalgae not only depends on the strain but also on the cultivation medium. For example, the coexistence of salts, heavy metals or non-metallic compounds often leads to complex interactions with each other and with living organisms, affecting their salt tolerance (Kumari et al., 2015). In comparison between the simpler and the complex media having a same salinity, the growth inhibition of green microalgae is often more significant in the latter (Arora et al., 2019; Sahle-Demessie et al., 2019). Unfortunately, the understanding of the effect of cultivation medium on the water desalination of microalgae is scarce and needs to be elaborated further.

In this study, the halophytic algae *Chlorella vulgaris* (*C. vulgaris*) was cultivated in a traditional medium (Bold Basal Medium) and a simulated Sea Salt medium at different sodium chloride contents using a Photobioreactor system in carefully controlled conditions (light density and air flow rate). Subsequently, the growth rate of microalgae and their salt absorption rate were continuously measured during 15 cultivation days to investigate the salt tolerance as well as the water desalination capacity of *C. vulgaris* in these media.

2. Materials and Methods

2.1. Materials

C. vulgaris was provided by the Laboratory of Microalgae (Nong Lam University - Ho Chi Minh City, Vietnam). Chemical substances and ocean salt (hw-Marinemix professional) were purchased from Xilong Scientific (China) and Wiegandt (Germany), respectively. The chemical composition of culture media was summarized in Table 1.

Table 1. Cultivation media composition

Bold Basal Medium (BBM)	
Chemical substances	Composition content
$NaNO_3$	25000 mg/L
$MgSO_4.7H_2O$	7500 mg/L
K_2HPO_4	7500 mg/L
KH_2PO_4	17500 mg/L
$CaCl_2.2H_2O$	2500 mg/L
H_3BO_3	114 mg/L
$EDTANa_2$	500 mg/L
KOH	310 mg/L
$FeSO_4.7H_2O$	498 mg/L
H_2SO_4	98 mg/L
Sea Salt Medium (SSM) at 30 mg/L	
Chemical substances	Composition content
Ca^{2+}	440 mg/L
Mg^{2+}	1320 mg/L
Na^{+}	10000 mg/L
Cl^{-}	20000 mg/L
$NaNO_3$	25000 mg/L
K_2HPO_4	7500 mg/L
KH_2PO_4	17500 mg/L

Source: Andersen (2004).

2.2. Sample preparation & experimental design

Firstly, the mother culture was grown in the Bold Basal Medium at 25°C for 15 days to achieve a microalgae density of 60×10^6 cells/mL. Then, the culture was transferred to photobioreactors (Figure 1) to investigate water desalination. The initial density of *C. vulgaris* for all the bioreactors was 10^6 cell/mL. For BBM, sodium chloride was added to the solution to reach the salt concentrations in the range at 1,000 to 15,000 ppm. For SSM, sea salt was diluted in water to adjust the concentrations in a range of 1,000 to 30,000 ppm. The photobioreactors were operated with a

light intensity of 4,000 - 8,000 Lux and an aeration rate of 4 L/min.

Figure 1. The photobioreactor system.

2.3. Microalgae growth measurement

The density of microalgae was estimated based on the combination of light microscopy and optical density (Barahoei et al., 2021). Firstly, microalgae were filled into a counting chamber (Marienfield, Germany) and observed under a light microscope (DM2500, Leica, Switzerland) equipped with a digital camera (DFC450C, Leica, Switzerland). Besides, the optical density (OD) of *C. vulgaris* at various concentrations was determined at 680 nm using a spectrophotometer (UV1100, MRC Lab, Israel) to build the growth curves for microalgae.

2.4. Salinity reduction measurement

Microalgae were collected on days 3, 6, 9, 12 & 15. For pre-treatment, samples were precipitated by using a benchtop centrifuge (Rotofix 32A, Hettich, Germany) at 4,000 rpm. Then the water salinity reduction was estimated using a conductivity meter (HI98318, Hanna, Rumani) and a salinity refractometer (SLI-10, China).

2.5. Statistical analysis

All measurements were triplicate performed. Analysis of variance (ANOVA) and Least Significant Difference (LSD) Test with 95% confidence was applied using the Statgraphics software (version 14) to investigate the effect of cultivation time and salt concentrations on the salt tolerance and the water desalination capacity of microalgae.

3. Results and Discussion

3.1. *Chlorella vulgaris* growth in different media

The growth of *C. vulgaris* in BBM and SSM at different salt concentrations after 15 cultivation days is depicted in Figure 2. In general, *C. vulgaris* showed a good growth at salt concentrations of 1 - 15 g/L. When the salt concentrations were greater than 20 g/L, the growth of *C. vulgaris* seemed to be inhibited and the medium color changed from green to yellow. A similar result was reported in a recent study of the salt tolerance of *C. vulgaris* (Sahle-Demessie et al., 2019). The authors suggested that at the high salt concentration (>10 g/L), nutrient deficiencies could occur and therefore microalgae must spend more time for synthesizing new chlorophyll molecules as well as binding new proteins.

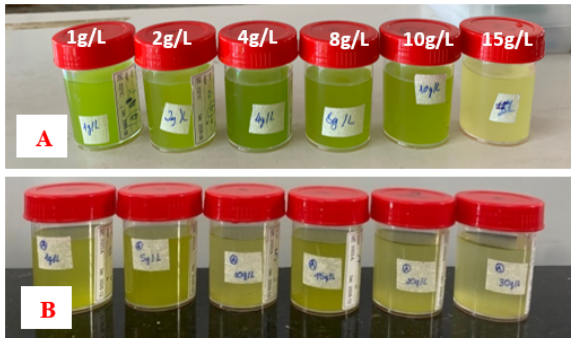

Figure 2. Microalgae at different salt concentrations. Bold basal medium (A), sea salt medium (B).

To estimate their growth curves, microalgae images were observed under a microscope (20 x) and the cell density was determined by combining an improved Neubauer counting chamber with ImageJ software (Figure 3). Meanwhile, the optical density of microalgae was also measured by a spectrophotometer at 680 nm (Barahoei et al., 2021). Obtained results revealed that *C. vulgaris* growth during the cultivation could be quickly estimated with a high reliability ($R^2 > 0.99$; Figure 4) using the optical density value based on the following equations:

$$\text{BBM: } y = 4.10^{-8}x + 0.0284$$
$$\text{SSM: } y = 3.10^{-8}x + 0.0652$$

with y being the absorbance value measured at 680 nM and x being the microalgae cell density.

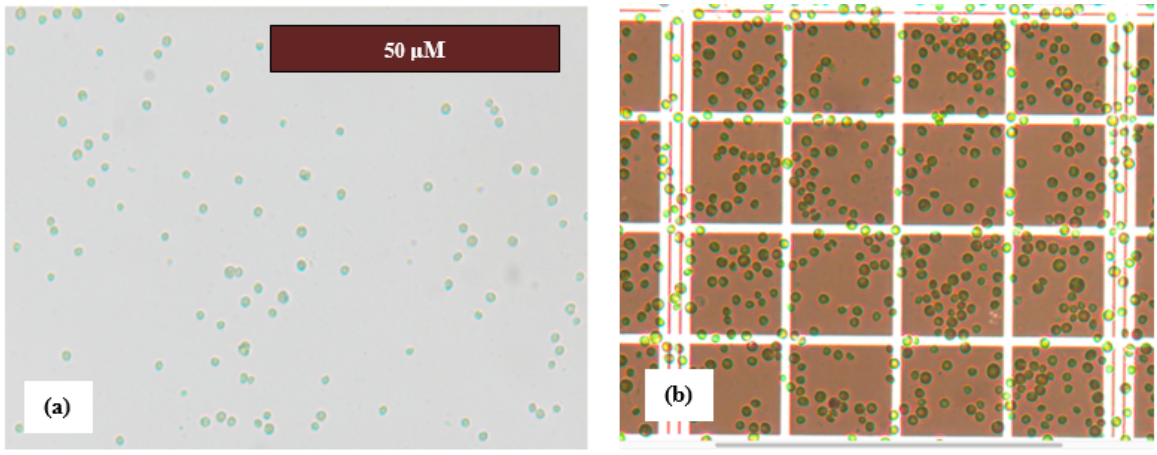

Figure 3. Microalgae images under the microscope with scale bar 50 µM (a) and the improved Neubauer counting (b) chamber.

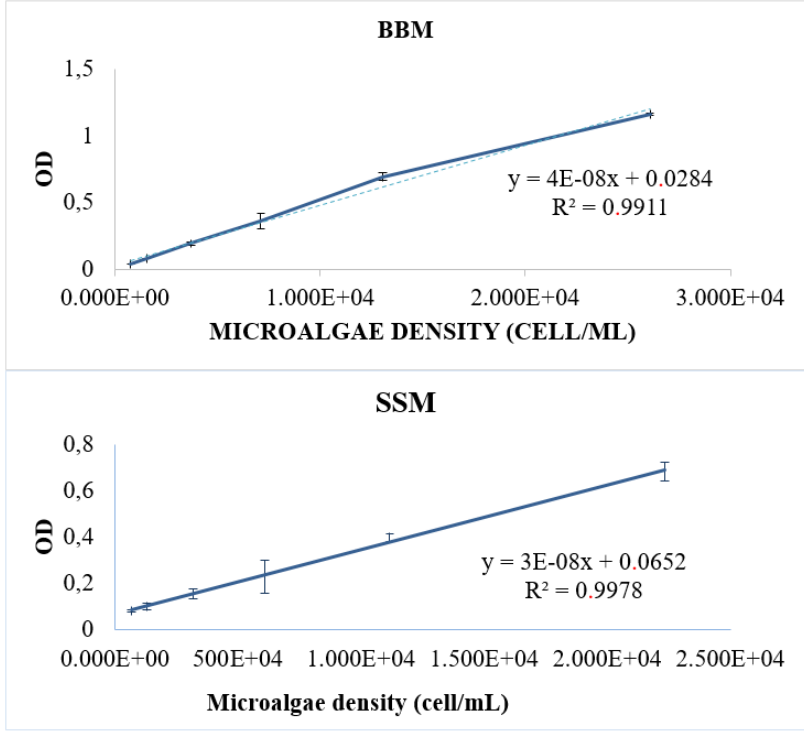

Figure 4. Standard curve for determination of algae density in bold basal medium (BBM) & sea salt medium (SSM) using spectrophotometer method.

The development of *C. vulgaris* cell density during 15 days of cultivation in BBM and SSM with different salt concentration was summarized in Figure 5. Similar to previous studies (Sahle-Demessie et al., 2019; Barahoei et al., 2021), our results revealed that microalgae showed good growth in BBM at most salt concentrations and reached 40 - 60 million cells/mL after 6 days of cultivation. Although the growth rate of microalgae decreased in the following stage, the cells were still alive in media having less than 15 g NaCl/L. As described in a previous section, the inhibition of *C. vulgaris* occurred when the salt content in BBM was over 15 g/L and a significant drop was recognized in the growth curve of microalgae.

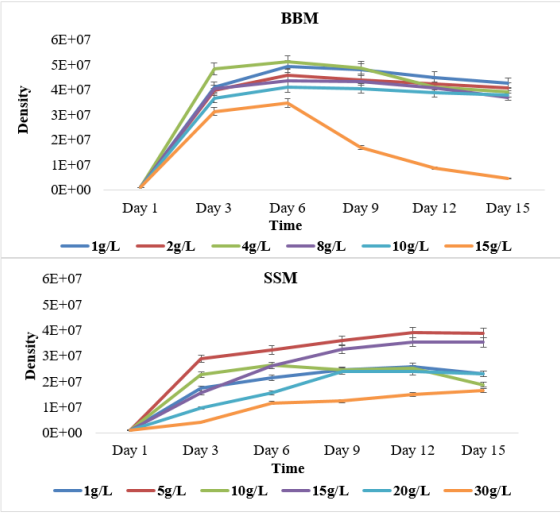

Figure 5. The growth curve of *C. vulgaris* in different media (bold basal medium-BBM & sea salt medium-SSM) over 15 days of cultivation.

For the SSM at most salt concentrations, *C. vulgaris* needed more time for growing and achieved the maximum cell density after 9 - 12 cultivation days. In comparison with BBM, the cell densities (number of cell/mL) of *C. vulgaris* cultivated in SSM are lower at the same salt concentration. This phenomenon is mainly related to the responses and adaptations of microalgae to environmental conditions (Hiremath & Mathad, 2010). According to many studies (Hasegawa, 2000; Hoque, 2007), the adaptation of eukaryotic organisms such as microalgae to adverse environmental conditions often results in changes in metabolism, synthesis or accumulation of some organic substances or osmosis behavior. When comparing BBM and SSM compositions, the latter had a lower content of nutritional compounds, and this could contribute to alleviating the growth rate of microalgae. On the other hand, BBM had higher ratios of Na^+/Ca^{2+} and Na^+/K^+ in comparison to SSM which could negatively affect the stress tolerance of plants with the change in environmental conditions. Therefore, the survival of *C. vulgaris* in SSM was still recognized at the very high salt concentration (20 - 30 g/L) while a significant decrease of microalgae was observed in BBM containing over 15 g NaCl /L.

3.2. Water desalination capacity of *C. vulgaris*

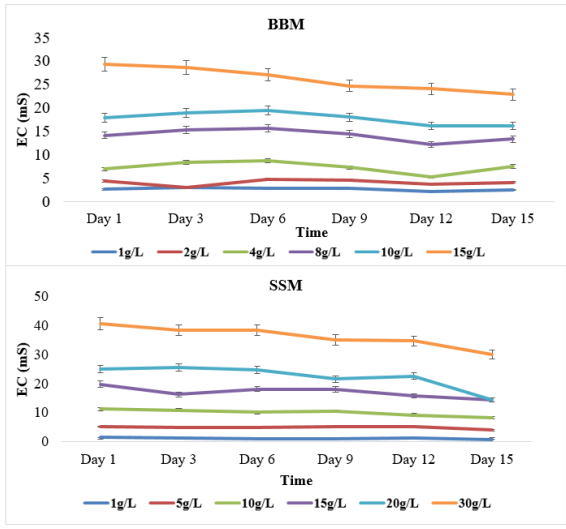

Figure 6. Electric conductivity (EC) changes in bold basal medium (BBM) & sea salt medium (SSM) over 15 days.

To estimate the water desalination, the electric conductivity and the salinity are the most popular indicators. These parameters were measured during the cultivation and the results were summarized in Figure 6 & 7. As expected, obtained results demonstrated that the salt content in water tended to decrease with the growth of microalgae. For instance, the salinity of BBM was reduced by 5% to 10% while the conductivity was reduced by about 2% to 20%. The 2 - way ANOVA analysis (Tables 2 & 3) also confirmed that salt concentration and water treatment duration were factors that significantly influenced the ability to reduce water salinity when using microalgae *C. vulgaris* ($P < 0.05$). Based on LSD

Table 2. Analysis of variance for electric conductivity

Bold basal medium					
Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Main effects					
A:Concentration	2464.89	5	492.979	277.67	0.0000
B:Time	38.8759	5	7.77518	4.38	0.0053
Residual	44.385	25	1.7754		
Total (corrected)	2548.15	35			
Sea salt medium					
Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Main effects					
A:Concentration	4967.18	5	993.435	294.44	0.0000
B:Time	102.136	5	20.4272	6.05	0.0008
Residual	84.3499	25	3.374		
Total (corrected)	5153.66	35			

Table 3. Analysis of variance for salinity

Bold basal medium					
Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Main effects					
A:Concentration	699.591	5	139.918	474.85	0.0000
B:Time	2.6721	5	0.53442	1.81	0.1466
Residual	7.3665	25	0.29466		
Total (corrected)	709.629	35			
Sea salt medium					
Source	*Sum of Squares*	*Df*	*Mean Square*	*F-Ratio*	*P-Value*
Main effects					
A:Concentration	2075.97	5	415.194	179.34	0.0000
B:Time	62.5487	5	12.5097	5.40	0.0017
Residual	57.8776	25	2.3151		
Total (corrected)	2196.4	35			

comparison, the desalination effect was more considerable in the medium containing a higher initial salt concentration ($P < 0.05$).

Similarly, the salinity of SSM decreased from 22% to 44% while the conductivity was reduced by about 23% to 43%. This result is in line with the finding in a recent study on the salt tolerance of *C. vulgaris* (Barahoei et al., 2021). The author reported that *C. vulgaris* microalgae could reduce about 45% of salt content in the sea salt culture medium with an initial concentration of 1 g/L - 5 g/L. The mechanism for salt adsorption in marine microalgae is not completely clear. It can be assumed that the cell wall of microalgae is a double enveloped membrane composed of phospholipids and plays an important role in exchanging metabolites and ions (Safi et al., 2013). When the salt concentration in aqueous solution is too high, anion Cl- tends to accumulate on the surface of the microalgae membrane and then attracts Ca^{2+}, Na^{+} & K^{+}. This phenomenon could contribute to accelerating the transportation of these ions through the cell membrane of microalgae resulting in a salinity reduction (Amezaga, 2014). Therefore, with a higher initial salt concentration in the cultivation medium, the salinity reduction should be more considerable. According to Figure 6 & 7, this trend was recognized for both media during the cultivation.

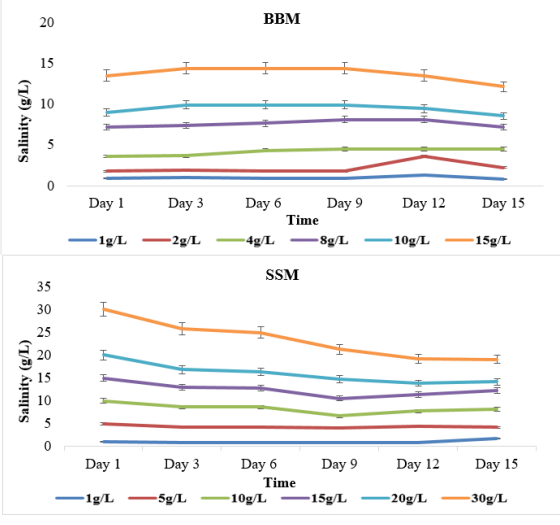

Figure 7. Salinity changes in bold basal medium (BBM) & sea salt medium (SSM) over 15 days

4. Conclusions

This study investigated the salt tolerance and water desalination capacity of the microalgae *C. vulgaris* in the traditional medium and the stimulated ocean medium. As expected, *C. vulgaris* showed good growth in both media and achieved 40 - 60 million cells/mL after 15 days of cultivation. For the salt tolerance, obtained results revealed that halophytic microalgae such as *C. vulgaris* could survive and develop in the sea salt medium with a salt content of up to 30 g/L. Based on electrical conductivity and salinity measurement, the growth of *C. vulgaris* contributed to the reduction of NaCl content in a range of 2 - 22% (for BBM) and 22 - 42% (for SSM). However, the study was conducted at the laboratory scale using small photobioreactors. Further studies, therefore, should focus on investigating and optimizing the water desalination capacity of *C. vulgaris* at the pilot scale using stirred tank reactors or tubular flow reactors.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgements

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Improving the database on land use planning in Vung Tau city, Ba Ria - Vung Tau province

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ABSTRACT

Vung Tau city is an area where management of land use planning (LUP) and urban planning is quite complicated and has been successful in building LUP database. However, this database still has limitations that must be addressed, including (1) lack of data layer on current land use for building LUP database, (2) incompleted LUP attribute database, (3) lack of metadata, (4) no connection between LUP database and digital legal materials and (5) no overlay cadastral database to data layer of current land use and LUP database. To solve these problems, this research used many methods such as documents and data collection, inheritance, expert interview, data processing and analysis, mapping, GIS application, and IT applications. The results of this study showed that the structure of cadastral database of 8 wards was succesfully converted from existing structure (according to Circular 17/2010/TT-BTNMT) to the standard structure (according to Circular 75/2015/TT-BTNMT). The 2019 land inventory data was standardized and data layer of current land use for whole city was built. In addition, the LUP database (period 2010 - 2020) for Vung Tau was improved, including: supplementing attribute information, completing metadata, connecting LUP database with relevant digital legal records, overlaying cadastral database with LUP database and data layer of current land use. Briefly, our results could be a foundation to help Vung Tau manage land sustainably, contribute to successfully building national land database and meet the exploitation requirements of LUP information in accordance with the orientation of Ministry of Natural Resources and Environment for study area.

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1. Introduction

Vung Tau city, Ba Ria - Vung Tau (BR-VT) province is an area where many projects are being implemented, so the demand for information about land and construction of people is extremely large. However, Vung Tau still does not have a complete land use database. Firstly, the operating cadastral database was built according to the old regulations in Circular No. 17/2010/TT-BTNMT and Circular No. 04/2013/TT-BTNMT. Next, the database of land use planning (LUP) has just been tested and showed many shortcomings, such as: (1) lack of data layer on current land use for building LUP database, (2) incompleted LUP attribute database, (3) lack of metadata, (4) no connection between LUP database and digital legal materials, (5) no overlay the cadastral database to data layer of current land use and LUP database. To expoit LUP database more effectively, it is very necessary to keep on improving LUP database in accordance with the active regulations for Vung Tau. It will be a useful tool in the land management of locality and the process of capturing LUP information of citizens, as well as meeting the requirement of exploiting LUP database in accordance with the orientation of MONRE.

2. Materials and Methods

Vung Tau archived full legal documents on the formulation of LUP (2010 - 2020) and adjustment of LUP (2016 - 2020). The works and projects in LUP (2010 - 2020) and LUP adjustment (2016 - 2020) are presented quite specific and exactly (PC of BR-VT, 2014 & 2019).

Vung Tau already had a testing LUP database (2010 - 2020), which was one of products from scientific project was appoved in 2021 (Truong, 2021). By using VBDLIS, study built a testing LUP database (2010 - 2020) for Vung Tau in accordance with MONRE regulations (MONRE, 2017 & 2018) with 6 data layers contains information about LUP, LUP adjustment and corresponding projects (QuyHoachSDDCapHuyen, CongTrinhDuAnCapHuyen, DieuChinh_QuyHoachSDDCapHuyen, DieuChinh_CongTrinhDuAnCapHuyen, DoiTuongThaiThamVan and YkienThamVan). Results of building testing LUP database on VBDLIS include (1) successfully built spatial database of LUP and adjusted LUP for whole city, (2) automatically imported LUP attribute data for whole city and (3) supplementing all data that have not been automatically imported for Ward 8, Vung Tau city with specific results (Table 1).

Table 1. Statistics on number of data records managed on land use planning (LUP) database (Unit: record)

No.	Data content	Vung Tau city	Ward 8
1	LUP	22,294	168
2	LUP project	164	8
3	LUP adjustment	14,650	700
4	LUP adjustment project	570	36

Source: Truong (2021).

2.1. Data collection and evaluation

With many methods (Vu, 2007) such as collecting documents and data (secondary and primary), select research site, inheritance, expert interview, data processing analysis, the study has collected full necessary documents and data for improving LUP database (period 2010 - 2020) of Vung Tau (LUP maps, LUP options and LUP database; cadastral database; 2019 land inventory data and other relevant documents and data). Results of analyzing and evaluating input data showed that data quality basically meets requirements of improving LUP database:

(1) Cadastral database is basically managed in accordance with regulations on management, exploitation, usage and updating cadastral databases (Table 2). However, this database was built before 2015 (Circular No. 17/2010/TT-BTNMT), and it is no longer consistent with land data standard in Circular 75/2015/TT-BTNMT. It has caused many difficulties for process of synchronizing databases at all levels and cannot be integrated into national land database (Truong, 2018), including LUP database. To help LUP database meet the requirements of MONRE, it is necessary to convert data structure of current cadastral database from existing structure to the standard one (MONRE, 2015).

Table 2. Statistics on number of data records managed on cadastral database

No.	Data content	No. of record
1	Land manager, land user and owner of property on land	102,139
2	Parcel	101,686
3	Property on land	72,529
4	Land use right certificate	98,050
5	Land use change registration	152,317
6	Records are conducted by electronic ISO process	116,486

Source: Land registration office of BR - VT province.

(2) The data source on current land use was dataset of 2019 land inventory and current land use map (PC of BR - VT, 2020). This dataset was basically complete, accurate, clear and consistent with MONRE regulations (MONRE, 2018) as shown in Table 3. However, land decamation layer on spatial data is incorrect (located at level 10 instead of level 5). Therefore, converting current land use data to the data standard of land inventory and statistics has been done previously (MONRE, 2015 & 2018). To successfully build data layer of current land use, it is necessary to carefully review and standardize this data source.

(3) Legal documents of LUP created a good support for process of digitizing records and connecting digital records into LUP database. The works and projects in LUP (2010 -2020) and LUP adjustment (2016 - 2020) are presented quite specific and exactly. They help process of supplementing content of LUP database to be more ad-

vantageous; and help all kinds of user easily exploit, access and look up information from this database (Truong, 2021).

Table 3. Statistics on number of data records in 2019 land inventory dataset

No.	Data content	No. of record
1	Area	17,747
2	Object code	17,747
3	Previous period's object code	6,594
4	Land type code	17,747
5	Previous period's land type code	6,594

Source: Land registration office of BR - VT province.

(4) LUP database just stopped at testing phase and remained many limitations such as: (1) lack of data layer on the current land use; (2) attribute database was incomplete because VBDLIS did not automatically import all input data into LUP database; (3) not connect LUP database with digital legal documents; (4) lack of metadata; (5) has not overlayed cadastral database to data layer of current land use and LUP database because the structure of cadastral database was incorrrect with the active land data standard (MONRE, 2015). This problem has caused a lot of difficulties in land management of the locality and the process of capturing LUP information of citizens. To improve LUP database for Vung Tau, it is very essential to overcome the above limitations.

2.2. Improving LUP database (period 2010 - 2020)

With GIS application method (Le, 2007), ArcGIS Desktop 10.1 was used to extract and review existing cadastral spatial data to well support a process of transforming this data into new structure (MONRE, 2015). MicroStation was used to review and standardize 2019 current land use map in accordance with prescribed standards (MONRE, 2018) for building data layer of current land use.

With method of applying Information Technology (Le, 2007), Microsoft SQL Server DBMS and ViLIS 2.0 were used to extract cadastral database and synthesize data for assessing the state of cadastral database. Many modules of VBDLIS were used to improve LUP database as: (1) LISEditorTC in converting cadastral spatial data, exporting electronic cadastral books and registration information; (2) VBDLIS.ConvertData in converting cadastral attribute data and connecting cadastral database to VBDLIS system; (3) QuyHoachKHSDD Desktop in building data layer of current land use, supplementing content of LUP database, completing metadata content, connecting LUP database with digital legal records, overlaying cadastral database with LUP database and data layer of current land use for whole city.

From results of the data input assessment, LUP database (2010 - 2020) was completed as shown in Figure 1.

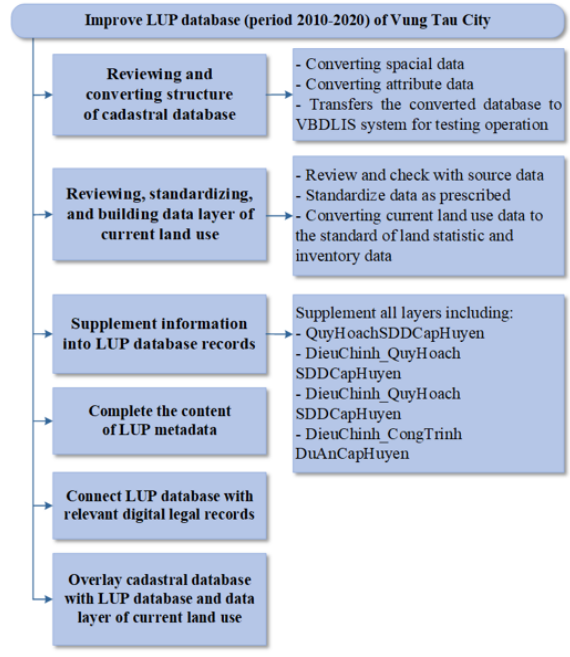

Figure 1. Process of improving the database on land use planning (LUP).

3. Results and Discussion

3.1. Cadastral database structure conversion

Process of converting cadastral database structure is carried out sequentially for each ward by 2 steps: converting spacial data as shown in Figure 2 and converting attribute data as shown in Figure 3.

By LISEditorTC Module of VBDLIS, cadastral spatial database of 8 wards has successfully been converted (Table 4). Result has created a

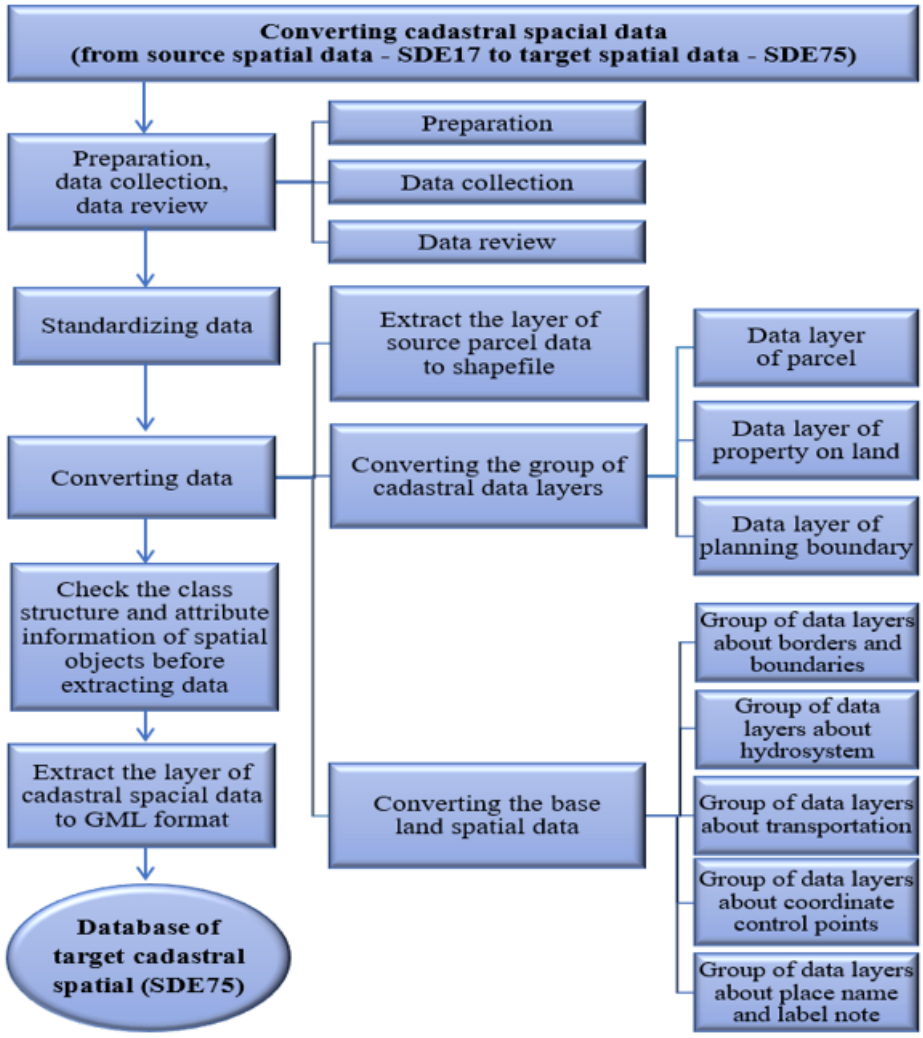

Figure 2. Process of converting cadastral spacial data.

SDE17: Existing cadastal spatial database was built according to Circular 17/2010/TT-BTNMT.
SDE75: New cadastal spatial database, which was converted to legal data standards in Circular 75/2015/TT-BTNMT.

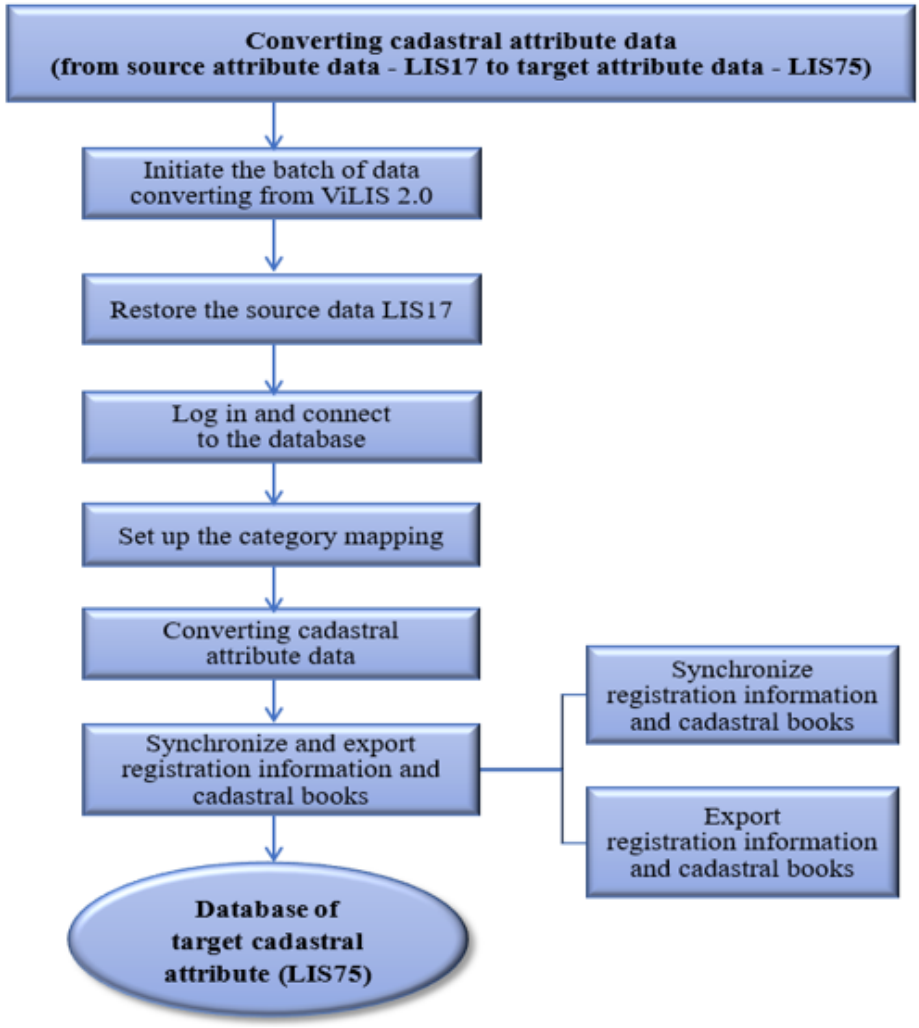

Figure 3. Process of converting cadastral attribute data.

LIS17: Existing cadastal attribute database was built according to Circular 17/2010/TT-BTNMT.
LIS75: New cadastal attribute database, which was converted to legal land data standards in Circular 75/2015/TT-BTNMT.

Table 5. Results of converting cadastral attribute data

No.	Administration unit	Parcel data (record)	Data on registration application (record)	Digital cadastral book (file)	Registration information in *.xmL format (file)
1	Ward 8	6,325	6,307	6,240	1
2	Ward 9	3,577	3,592	3,575	1
3	Ward 10	6,467	7,004	6,472	1
4	Ward 11	4,941	4,851	4,938	1
5	Ward 12	13,565	13,549	13,563	1
6	Nguyen An Ninh	3,658	4,026	4,035	1
7	Rach Dua	5,949	5,940	5,947	1
8	Thong Nhat	4,037	4,059	4,037	1
	Total	48,519	49,328	48.807	8

Table 4. Results of converting cadastral spatial data

No.	Administration unit	Base land spatial data (record)	Cadastral spatial data (record)	Cadastral spatial data in *gmL format (file)
1	Ward 8	549	10,046	13
2	Ward 9	631	4,996	13
3	Ward 10	632	9,407	13
4	Ward 11	1,094	11,385	13
5	Ward 12	2,063	17,691	13
6	Nguyen An Ninh	379	5,029	13
7	Rach Dua	555	9,180	13
8	Thong Nhat	985	12,937	13
	Total	6,888	80,671	104

cadastral spacial database which is accurate, synchronous, adaptable with legal standard and well supports the process of improving LUP database.

The conversion process of cadastral attribute data (from LIS17 to LIS75) is conducted by data conversion tool of VBDLIS. This work requires to be carried out carefully and in the right order to ensure high accuracy and reliability in mapping process of each record (Table 5).

Then, study transfers converted database to VBDLIS system for testing operation via https://dt.mplis.gov.vn (Figure 4) to serve multiple purposes in land management. Because all land information will be updated fast, easy and timely as well as securely stored for minimizing possibility of data leakage, due to less affection from objective factors. Database security will be much higher than traditional method. Achieved results will support locality in managing, exploiting, using and sharing of cadastral database in an effective way; and facilitating process of improving LUP database, successfully building land database for BR - VT and nationwide.

3.2. Building the data layer of current land use

The current land use data plays an important role in data building for many purposes such as: (1) generalize results of land statistics and inventory on map; (2) basic documents for territorial and land management; (3) priority documents on serving formulation of LUP and inspection of approved LUP implementation process. To successfully improve and operate LUP database, it is very crucial to build a data layer of current land use (Figure 5).

By VBDLIS, data layer of current land use has successfully built with sufficient data (spatial and attributes) and in accordance with data standard, ensures the accuracy and necessary quality. The results serve well the process of integrating and improving LUP database at locality (specifically in Table 6; Figure 6):

3.3. Inserting data into the LUP database

From existing LUP database, to improve and exploit LUP database effectively, study supplement nescessary contents into all records that were not automatically imported by VBDLIS (Truong, 2021) (Figure 7).

After all, LUP database contains full information (spatial and attribute) in accordance with approved map and LUP with high accuracy and consists of 4 data classes as shown in Table 7.

3.4. Completing the content of LUP metadata

The LUP metadata helps to describe the most complete and detailed description of data and quality of data which is contained in LUP database, served effectively for process of exploiting, operated and shared LUP database. Study has supplemented 65 records of metadata for LUP data (Figure 8), ensured to describe completely about LUP data as regulation, and contributed to improving legality and quality of LUP database.

3.5. Connecting LUP database with relevant digital legal records

Connecting LUP database with relevant digital legal records to support users to look up 3 blocks of LUP information simultaneously (spatial, attributes, original record image). All digital legal records related to formulation and management of LUP have been successfully connected to LUP database (Figure 9).

The results support users to look up information comprehensively, help to strengthen legal value of LUP database and increases user's trust in the information which accessed from database, reduce time of searching related information, improve the rigor, and ensure the publicity and transparency in process of implementing LUP at locality.

3.6. Overlay cadastral database with LUP database and data layer of current land use

Overlaying cadastral database with LUP database and data layer of current land use helps users look up LUP information comprehensively (cadastral, land use status, LUP) and the management of LUP be more quickly, accurately, conveniently and transparently (Figure 10).

Except LUP information, users also look up more information about cadastral and current land use of parcel affected by LUP option (username, land parcel ID, address, area, current land use); help to exploit and querry LUP information more comprehensively, meet the need of diversity objects in land transactions and looking

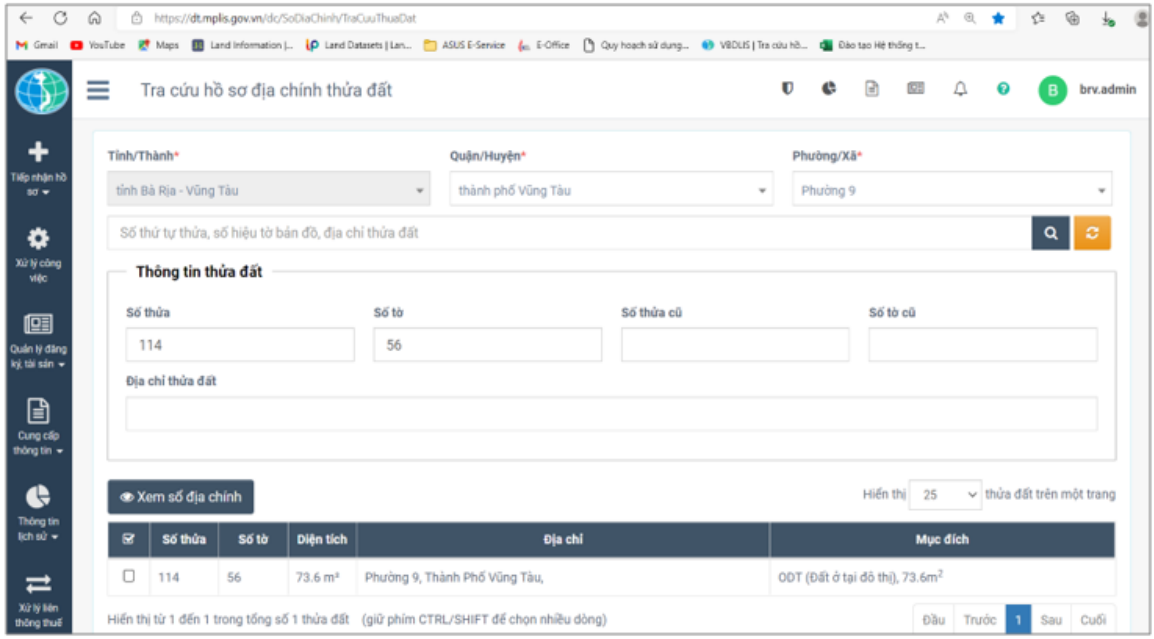

Figure 4. Result of importing the completed cadastral database to VBDLIS system.

Source: https://dt.mplis.gov.vn/.

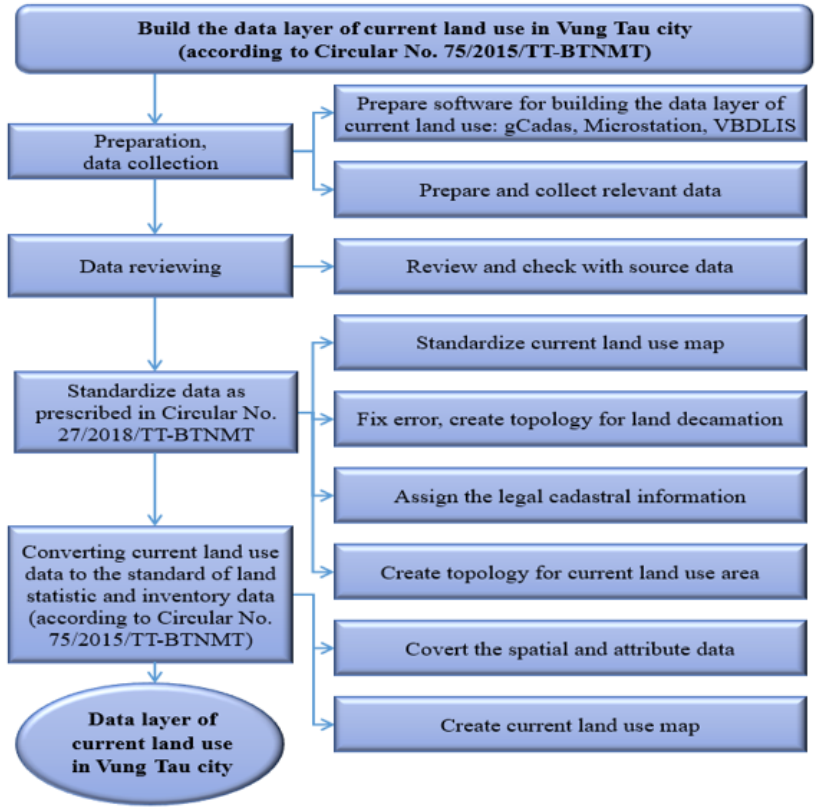

Figure 5. Process of building data layer of current land use.

Table 6. Result of building data layer of current land use

No.	Administration unit	No. of land demarcation	Area (ha)
1	Ward 1	511	178.18
2	Ward 2	536	293.68
3	Ward 3	818	90.04
4	Ward 4	400	80.92
5	Ward 5	1,345	399.81
6	Ward 7	440	162.49
7	Ward 8	613	198.93
8	Ward 9	213	329.57
9	Ward 10	1,053	404.03
10	Ward 11	1,528	1,040.19
11	Ward 12	3,600	3,708.93
12	Nguyen An Ninh	642	480.07
13	Rach Dua	744	610.65
14	Thang Nhat	1,540	859.76
15	Thang Nhi	654	273.46
16	Thang Tam	581	249.92
17	Long Son	2,535	5,728.97
	Total	17,747	15,089.60

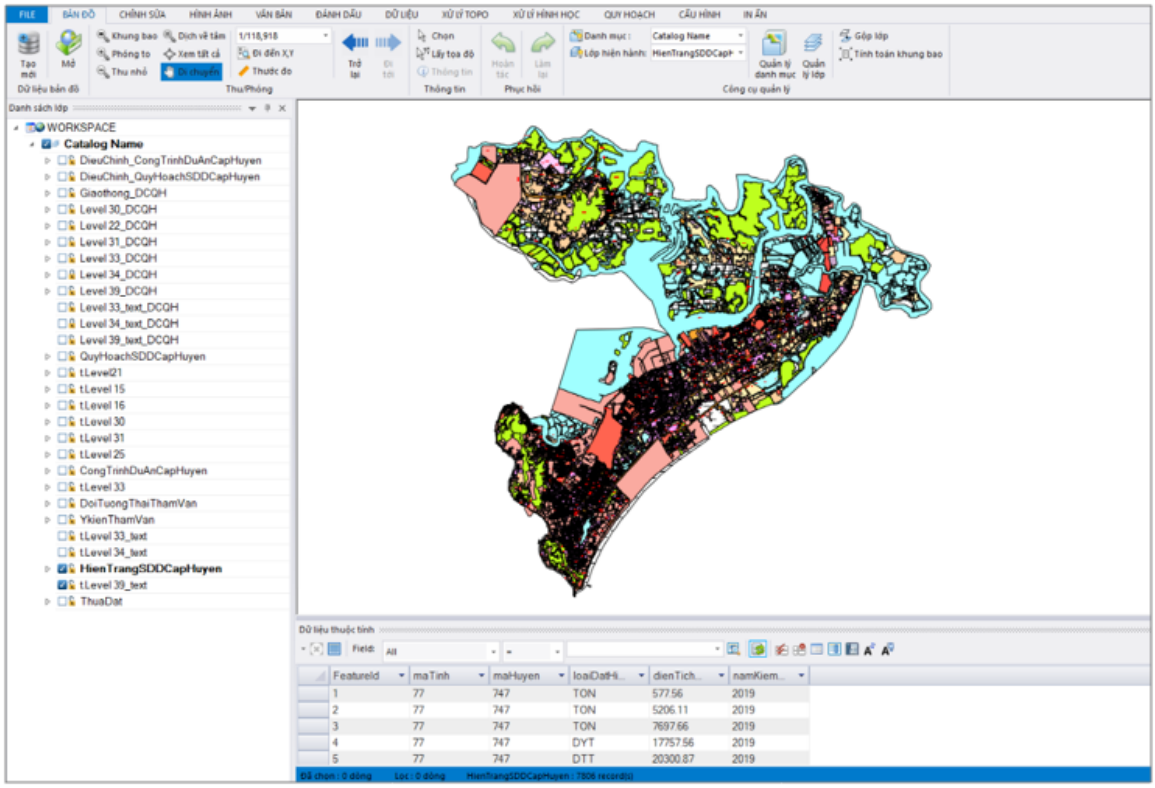

Figure 6. Results of building and overlaying data layer of current land use.

Table 7. Results of inserting data into the land use planning (LUP) database

No.	Name of data layer	No. of record
1	QuyHoachSDDCapHuyen layer	2,760
2	CongTrinhDuAnCapHuyen layer	37,580
3	DieuChinhQuyHoachSDDCapHuyen layer	4,054
4	DieuChinhCongTrinhDuAnCapHuyen layer	2,887
	Total	47,281

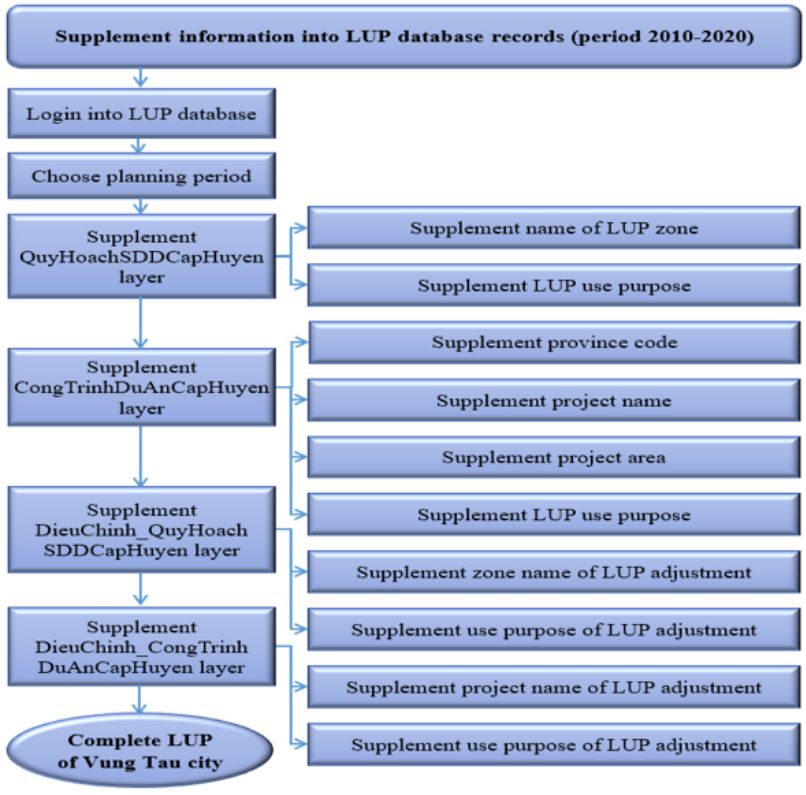

Figure 7. Process of supplementing information into land use planning (LUP) database.

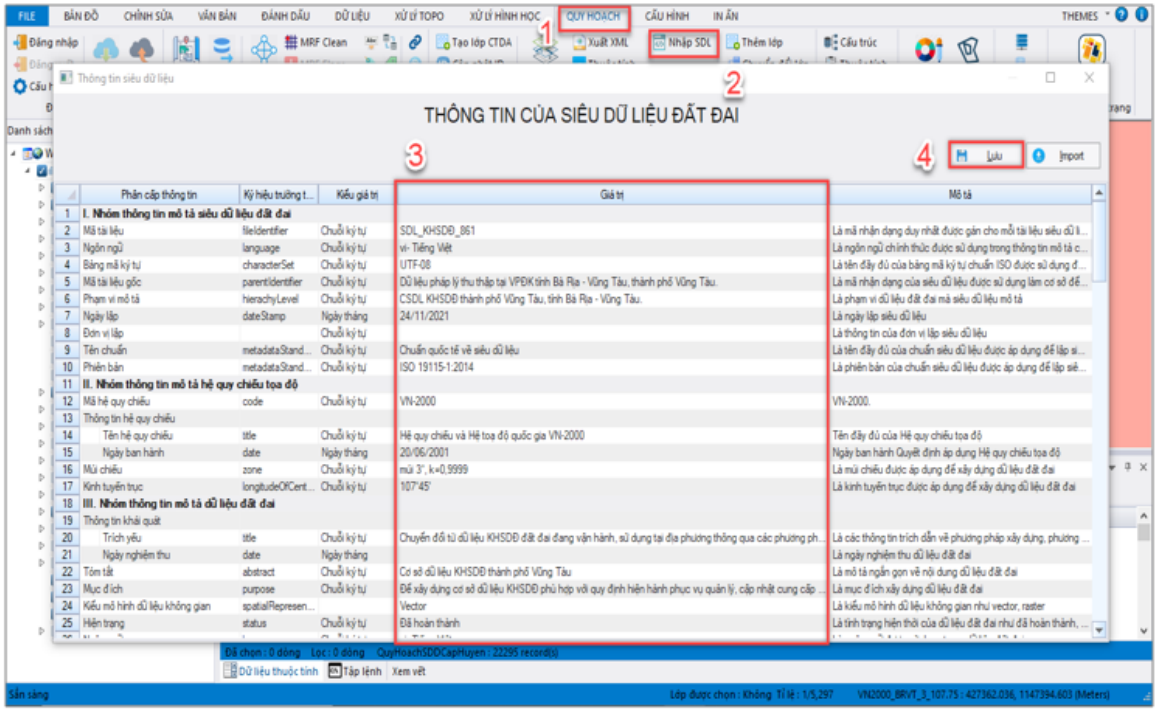

Figure 8. Results of completing the content of land use planning (LUP) metadata.

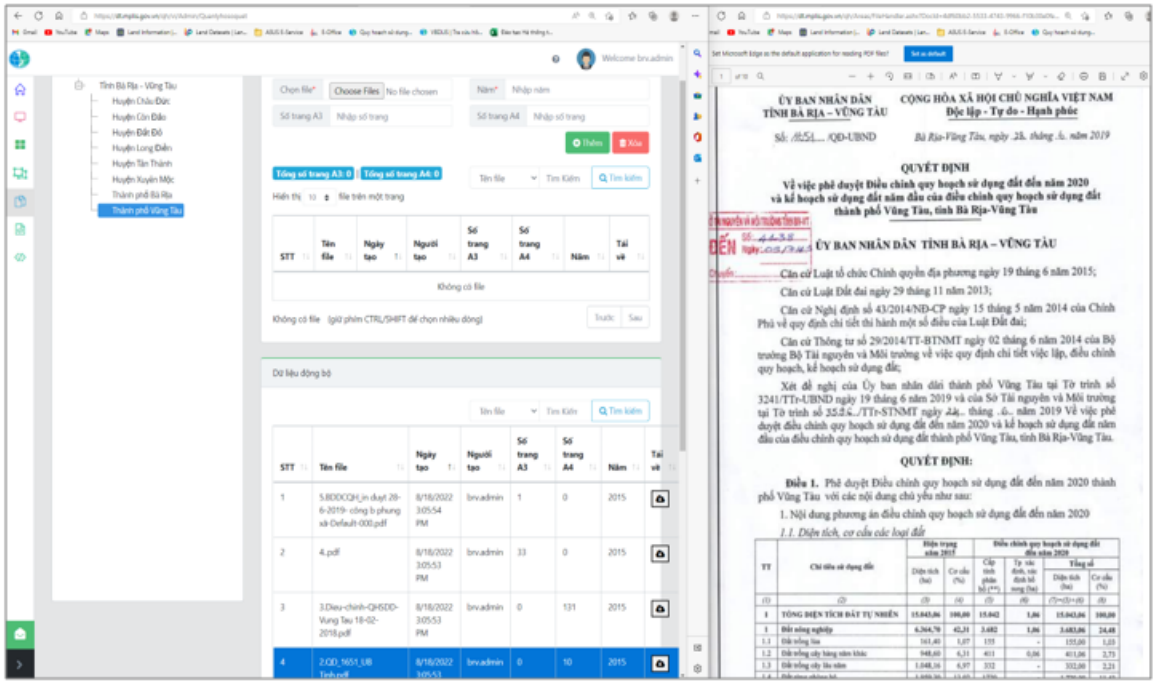

Figure 9. Resulst of connecting land use planning (LUP) database with relevant digital legal records.

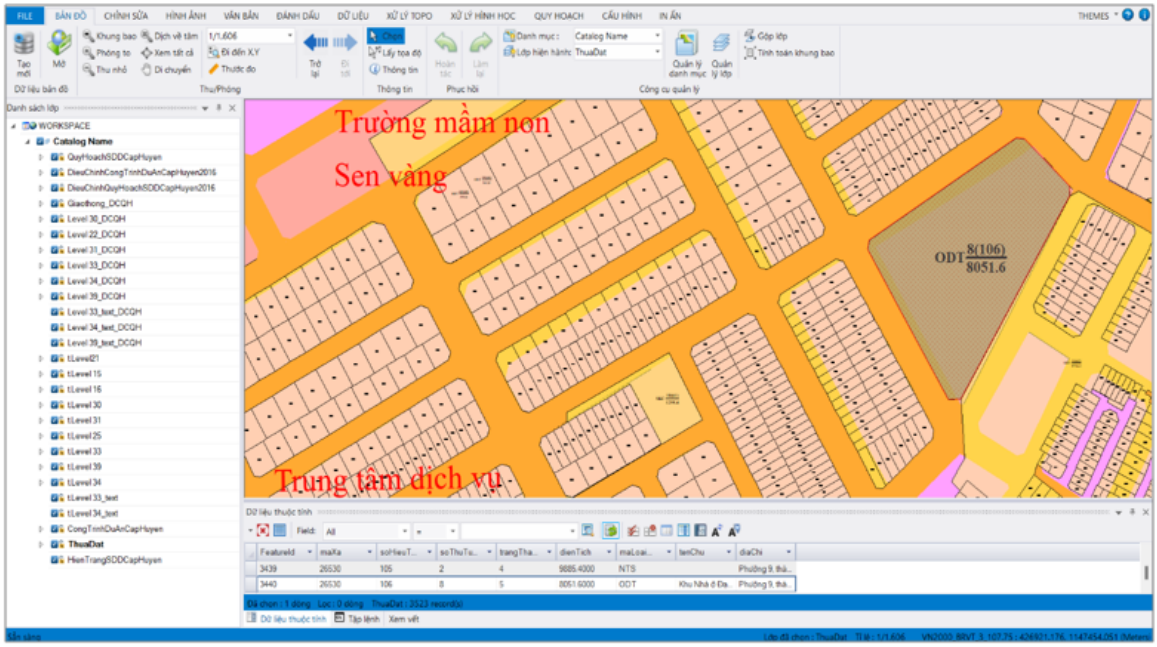

Figure 10. Result of overlaying cadastral database with land use planning (LUP) database and data layer of current land use.

up LUP information; and contribute to improving the publicity, transparency and reliability of citizens on decisions of local authority.

3.7. Discussion

From 1993 to 2021, in Vietnam, there were many studies around application of science and technology in formulating and managing LUP. That is scientific basis for perfecting policies and serving process of LUP modernization. However, those researches stop at applying information technology into some phases of data calculation and map digitization, there is no synchronous solution for database establishment and exploitation. The promulgation of standards for land databases, including standard for LUP databases (TCVN 13343:2021) mainly serves the goal of building a national land database (MOST, 2021). Many experimental studies on building district-level LUP database were carried out based on LUP options of respective period, but were incomplete, did not meet current data standards and existed many inadequacies in terms of content, data standards, infrastructure for management, exploitation and sharing database (Tran & Le, 2010; Doan et al., 2017; Nguyen et al., 2019). Researches on innovation and improvement of LUP processes, complete software to support development of LUP plans, GIS application and multi-criteria analysis methods to support selection of optimal LUP option (Nguyen et al., 2006; Nguyen, 2019; Pham, 2020)... have also been implemented in many places. However, these studies mainly focused on construction of a set of land use indicators in LUP at all levels and development of a set of economic, social and environmental criteria to evaluate effectiveness of LUP option for each level.

Up to 2021, "Research to design the model of LUP database at district level which is associated with the community consultation factor in Vung Tau city, BR - VT Province" (Truong, 2021) was the first work in Vietnam that addresses the issue of building LUP database in accordance with national database standards and mentioning to community consultation factors in LUP. Author analyzed the importance of community consultation in State management in Vietnam (especially in land management and LUP); and designed database model of LUP, which was associated with the community consultation factor, for Vung Tau in accordance with land data standards of Circular 75/2015/TT-BTNMT; successfully built a set of LUP database structure (with 22 spatial data tables on ArcGIS and 8

attribute data tables associated with the community consultation factor, on Microsoft SQL Sever), which was in high-precision and in line with data standards of MONRE and designed database model. By VBDLIS, study successfully build LUP database (period 2010 - 2020) for Vung Tau with 6 data layers: LUP data layer (15,060 records), project layer (163 records), adjustment LUP layer (12,002 records), adjustment project layer (570 records), and 2 data layers of community consultation. Results showed that the corrected model and completed database structure set were the basic for successfully building and effectively exploiting LUP database. This was the premise to implement land manage in accordance with approval LUP and improve land use efficiency in locality. However, this study needs to be further improved because the results obtained are only at experimental stage. This test LUP database of Vung Tau still has many limitations, has not fully met regulations of MONRE and actual exploitation needs in locality.

Together with priority development strategy of Viet Nam to be perfect and modernize land management industry with main goal is to build a complete national land use database (TECOVCP, 2022), it is very necessary to study and improve LUP database in accordance with national land data standards and associated with community consultation to improve the feasibility and effectiveness of LUP option. Closely linking LUP with community consultation factors will create conditions for citizens to boldly participate in contributing more ideas to process of making and managing LUP; help to increase the publicity, transparency and feasibility of LUP option; successfully building a modern, effective and efficient electronic land management system.

In general, results of this study have addressed the remaining problems in test LUP database of Vung Tau, proved the correctness and urgency in policy and orientation of MONRE on building a synchronous and unified national land database, which based on completing component databases (including cadastral database, land inventory and statistics database, LUP database and land price database, ...), (MONRE, 2015 & 2017). It creates a good condition to establish successfully land database and support process of administrative reform through online public services on land. It will help to meet the requirement of exploiting information from land and LUP in accordance with orientation of MONRE for the study area. These results can become useful references for further studies and be a basis for duplicating the model to other localities which have similar conditions.

To exploit the improvement of LUP database effectively and meet requirements of integrating and building land database in accordance with current regulations and directions for sustainable land use and management (TECOVCP, 2022), it is necessary to implement relevant further studies, including:

(1) Build models that help manage, operate, share and integrate LUP database into land database (MONRE, 2017) in the study area as LUP information system, provincial land information system, LUP database management, user management, exploitating and operating LUP database, sharing LUP database and integrating LUP database into land database.

(2) Supply classes of approved LUP (period 2020 - 2030), including mention to community consultation factor in LUP.

(3) Evaluate the status of community consultation in process of making and implementing LUP plan (period 2020 - 2030) in Vung Tau.

(4) Deploy to collect comments of local community and developing tools to collect, synthesize and process community consultation information to complete data layer of community consultation in making and managing LUP (period 2020 - 2030).

(6) Keep researching to establish land database model based on building and completing the remaining component databases for Vung Tau includes cadastral database, database of land statistic and inventory, land price database, land resource database... (MONRE, 2015).

4. Conclusions

The study has succeeded in addressing limitations of LUP database (2010 - 2020) for Vung Tau, as: (1) converting data structure of cadastral database; (2) building data layer of current land use; (3) supplementing 47,281 missing records of approved LUP into LUP database; (4) supplementing 65 metadata records; (5) connecting digital legal file to LUP database; (6) overlaying cadastral database with LUP database and data layer of current land use. Obtained results help Vung Tau to get completed LUP database in both structure and content according to the current

regulations, which helps process of exploiting and querrying LUP information to be more comprehensively, meets the needs of users in land transaction and looking up information... This is an important premise to build a land database for the locality and sychronize with land database at all levels. LUP database is a useful tool to support locality in managing and implementing LUP option, towards sustainable land management. The method of exploiting LUP database on network environment of VBDLIS system helps to improve the publicity and transparency of LUP plan and create good condition for citizens in grasping information when needed. It creates a favorable and healthy environment of real estate investment, improves people's living standards and contributes to the development of real estate market and local economy.

Conflict of interest

The author has no conflicts of interest to declare.

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