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CONTENT

Breeding new F_1 hybrids cucumber for the Southeastern market in Vietnam

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ABSTRACT

The objective of this study was to create new F1 cucumber varieties that would meet the demands of the regional market, in term of higher yield and reasonable cost. The study was conducted using 8 hybrid combinations: C2 x C44; C4 x C37; C12 x C44; C20 x C40; C31 x C44; C35 x C44; C41 x C52; C44 x C52 and a control variety Hunter 1.0. The growth time of hybrid combinations ranged from 64.3 to 66.7 days after sowing. The yield of the hybrid combinations ranged from 37.2 to 39.8 tons/ha. The hybrid combinations C20 x C40; C31 x C44, and C44 x C52 had a higher yield than Hunter 1.0 control. The fruit was from 17.9 to 19.7 cm long and the average weight per fruit was 156.7 - 193.3 g. The fruit flesh hardness of the hybrid combinations ranged from 30.9 to 32.0 lbf and was equal to or higher than that of the control variety.

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1. Introduction

Cucumber is a popular vegetable in Vietnamese people's daily meals. It is used to eat fresh or cooked, combining with salads, stir-fried dishes, etc. (Malepszy, 1988). With an ingredient that accounts for more than 95% of water, cucumber provides water, detoxification and moisture supply for the body (Liang et al., 2014). Moreover, cucumber also works to beautify the skin, help to lose weight, regulate cholesterol in the blood, cure constipation, etc. In addition, cucumber products are also processed canned in industry and export (Calbom & Calbom, 2008). According to the GOS (2010), the total cucumber growing area is 31,570 ha in Vietnam with an average yield of 18.3 ton/ha, which is higher than the average yield of the world.

The Southeastern in Vietnam has a favourable geographical location, concentrating on many major provinces and cities which is an important point of exchange and trade between the

Southern region with the whole country and the world (Dang et al., 1997). Agricultural land is a strong point of the region with a total land bank of 27.1% being used for agricultural purposes, of which cucumber is a strong crop (Duc, 2009). Based on the suitable climate and soil conditions, and skilled farmers with deep experience in growing cucumbers, the Southeast is the region that supplies a large number of cucumbers to the country and especially Ho Chi Minh City, where has the highest demand in the consumption of fruit and vegetables (Cadilhon et al., 2006).

Currently, farmers in the Southeast are mainly cultivating by a cucumber variety of Hunter 1.0 with outstanding features of good growth. The morphological characteristics of the fruit are suitable to regional tastes. This is the seed imported by East-West Seed Company, Vietnam, with a high selling price. However, the yield of this variety is not high, and the seed quality is not stable. The rate of infection with powdery mildew is high in the rainy season. Stemming from these situations, the market needs to have domestic cucumber varieties that are improved to replace this variety, providing high yield, good quality, strong plants, and well adapted to the natural conditions of the region.

Besides, in order to meet the needs of domestic production and consumption of seedlings, in September 2016, the People's Committee of Ho Chi Minh City decided to approve the "Program on developing high-quality variety and seedling in Ho Chi Minh City from 2016 through 2020" (PCHCM, 2016).

The objective of this study was to create new F1 cucumber varieties that would meet the demands of the regional market in term of higher yield and reasonable cost with the following characteristics: High productivity reaches over 35 tons/ha/crop; Fruit length is 18 - 20 cm, straight, balanced; Color: blue is darker than the left colour of Hunter 1.0; Fruit diameter < 3.5 cm. The rate of marketable fruits $> 90\%$; Crunchy, sweet equivalent to Hunter 1.0, not bitter (sensory evaluation).

2. Materials and Methods

2.1. Material

Eight cucumber hybrids bred by the dialling method were selected and developed from 12 varieties of S_7 generation with the agronomic characteristic was shown in Table 1, which was saved at the Research Center of Tan Loc Phat Seeds Company Limited. The Hunter 1.0 variety distributed by East-West Seed Company was used as the control variety in the study.

2.2. Methodology

2.2.1. Experimental design

Breeding F_1 hybrid from 12 S_7 cucumber lines by diallen method obtained 66 F_1 cucumber hybrid combinations. By comparing and evaluating the agronomic characteristics of the hybrid combination, the eight most promising hybrid combinations have been selected which were suitable for the market requirements.

This single factor experiment was arranged in a randomized complete block design, including nine treatments (8 hybrid combinations and one control variety - Hunter 1.0); 3 repetitions. The area of each experimental plot is 2.4 m x 1.2 m = 2.88 m^2. The planting distance is 1.2 x 0.6 cm. Applied technical procedures and evaluation criteria and monitoring methods are based on National Technical Regulations (MARD, 2012-QCVN 01-87:2012/BNNPTNT) on the value of cultivation and use of cucumber varieties by the Ministry Issued by Agriculture and Rural Development.

2.2.2. Monitoring criteria

Criteria of fruit morphology, yield and quality included yield, fruit length, fruit breadth, the average weight per fruit, rate of marketable fruits, fruit shape, fruit peel colour, fruit peel features and fruit hardness.

2.2.3. Statistical analysis

Data of experiments were collected and calculated on computers according to ANOVA statistical analysis method and LSD ranking test by using SAS software 9.1.

2.2.4. Time and location

The experiment was conducted in 9 months (from November, 2018 to August, 2019) at the Seeds Research Center of Tan Loc Phat Seeds Company Limited that is located in Ham Tan district, Binh Thuan province.

Table 1. Morphological characteristics of parental lines

Parental lines	Fruit shape	Fruit colour	Fruit peel features	Fruit length (cm)	Fruit diameter (cm)
C2	Cylinder, conical top, tapering bottom	Green, short stripes	Soft, white, thin spines , many pollens	17.8	3.4
C4	Cylinder, conical top, circular bottom	Green, short stripes	Soft, white, thin spines, fewer pollens	18.2	3.6
C12	Cylinder, conical top, spherical bottom	Light green, short stripes	Soft, white, thin spines, fewer pollens	19.2	3.5
C20	Cylinder, conical top, spherical bottom	Green, short stripes	Hard, white, thin spines, fewer pollens	17.8	3.5
C21	Cylinder, tapering top, circular bottom	Dark green, no stripes	Soft, white, thin spines, fewer pollens	18.7	3.6
C31	Cylinder, conical top, circular bottom	Green, fewer stripes	Hard, white, thin spines, fewer pollens 17.5	3.5	
C35	Cylinder, conical top, spherical bottom	Light green, short stripes	Soft, white, thin spines, many pollens	18.3	3.7
C37	Cylinder, conical top, tapering bottom	Light green, long stripes	Soft, white, thin spines, fewer pollens	17.6	3.5
C40	Cylinder, conical top, spherical bottom	Green, fewer stripes	Soft, white, thin spines, fewer pollens	19.4	3.4
C41	Cylinder, conical top, spherical bottom	Green, short stripes	Soft, white, thin spines, fewer pollens	20.1	3.6
C44	Cylinder, conical top, circular bottom	Green, short stripes	Soft, white, thin spines, fewer pollens	19.6	3.6
C52	Cylinder, conical top, spherical bottom	Green, fewer stripes	Soft, white, thin spines, fewer pollens	17.8	3.6

Table 2. Flowering periods and growth time of the hybrid combinations

Parental lines	Days to female flower initiation	Days to male flower initiation	Growth time (Days)
C2 x C44	26.5	25.4	64.7
C4 x C37	30.9	29.8	64.3
C12 x C44	31.5	27.3	65.3
C20 x C40	31.6	27.8	66.0
C31 x C44	28.3	27.1	66.3
C35 x C44	28.0	26.0	65.7
C41 x C52	28.7	25.9	65.0
C44 x C52	26.8	24.1	66.3
Hunter 1.0	31.7	27.8	71.3

Table 3. Morphological and yield characteristics of the hybrid combinations

Parental lines	Fruit length (cm)	Fruit diameter (cm)	Average weight per fruit (g)	Rate of marketable fruits (%)	Yield (ton/ha)
C2 x C44	18.0^{cd}	3.5^{b}	183.3^{ab}	97.1^{a}	37.2^{c}
C4 x C37	17.9^{cd}	3.5^{b}	166.7^{bc}	97.4^{a}	37.6^{c}
C12 x C44	19.3^{ab}	3.6^{a}	193.3^{a}	96.7^{a}	38.0^{bc}
C20 x C40	18.3^{bcd}	3.4^{b}	156.7^{c}	96.8^{a}	39.8^{a}
C31 x C44	18.4^{bcd}	3.5^{b}	186.7^{ab}	97.4^{a}	39.2^{ab}
C35 x C44	18.7^{abc}	3.6^{a}	173.3^{abc}	97.2^{a}	38.0^{bc}
C41 x C52	19.7^{a}	3.6^{a}	173.3^{abc}	97.2^{a}	38.3^{abc}
C44 x C52	18.5^{bcd}	3.6^{a}	173.3^{abc}	96.5^{a}	39.2^{ab}
Hunter 1.0	17.6^{d}	3.5^{b}	180.0^{ab}	95.0^{b}	37.6^{c}
CV%	2.2	0.8	6.1	0.8	2.1
Fvalue	7.74^{**}	16.0^{**}	3.15^{*}	2.83^{*}	3.49^{*}

In the same column, numbers with the same letter do not have a statistically significant difference; *: significant difference at the level of $\alpha = 0.05$; **: very significant difference at the level of $\alpha = 0.01$.

3. Results

As shown in Table 2, the deviation between male and female flowering date affects the pollination rate of the hybrid combinations. In terms of growth time, the combination C31 x C44 and C35 x C44 had the longest growing days and hybrid combination C4 x C37 had the shortest number of growing days with 65.7 to 66.6 days and 64.3 days, respectively. In general, the days to flower initiation in the hybrid combinations were earlier than the existing varieties in Vietnam's Southeastern market, with the flowering time ranging from 32 to 34 days after sowing.

In the Table 3, the C41 x C52 hybrid combination had the longest fruit length (19.7 cm) and was significantly different from the other hybrid combinations and the control variety. Furthermore, all hybrid combinations gave a longer length of fruit than varieties in the Vietnam's Southeastern market (Table 5) except for the C4 x C37 hybrid. Fruit breadth of the hybrid combinations C12 x C44, C35 x C44, C41 x C52, and C44 x C52 had the largest value (3.6 cm) and the difference is statistically significant compared to other treatments as well as four available varieties in the Southeastern market.

Cucumber's fruits in the C12 x C44 hybrid combination yielded the heaviest weight at 193.3 g per fruit, which was a higher and statistically significant difference compared to the C4 x C37 and C20 x C40 hybrid combinations. Moreover, this weight was also higher than the varieties as Hunter 2.0, SL 1.2 and Vino 67 on Vietnam's Southeastern market with an average weight per fruit similar to Hunter 1.0. The marketable rate of cucumber in the hybrid combinations showed high results and the difference was not statistically significant. All the hybrid combinations in the experiment had higher marketable fruit rate

Table 4. Fruit characteristics of hybrid combination

Fruit hardness (lbf)	Fruit peel features	Fruit peel color	Fruit shape	Parental lines
C2 x C44	Cylinder, conical top, circular bottom	Green, short stripes	Soft white spines, thin spines, fewer pollens	31.1^{cd}
C4 x C37	Cylinder, conical top, spherical bottom	Green, fewer stripes	Soft white spines, thin spines, fewer pollens	32.0^{a}
C12 x C44	Cylinder, conical top, circular bottom	Green, fewer stripes	Soft white spines, thin spines, medium pollens	32.0^{a}
C20 x C40	Cylinder, conical top, spherical bottom	Green, short stripes	Soft white spines, thin spines, medium pollens	31.3^{bcd}
C31 x C44	Cylinder, conical top, circular bottom	Green, fewer stripes	Soft white spines, thin spines, fewer pollens	30.9^{d}
C35 x C44	Cylinder, conical top, circular bottom	Green, short stripes	Soft white spines, thin spines, fewer pollens	31.5^{bc}
C41 x C52	Cylinder, conical top, spherical bottom	Green, fewer stripes	Soft white spines, thin spines, fewer pollens	31.4^{bc}
C44 x C52	Cylinder, conical top, circular bottom	Green, fewer stripes	Soft white spines, thin spines, medium pollens	31.6^{ab}
Hunter 1.0	Cylinder, conical top, circular bottom	Green, fewer stripes	Soft white spines, thin spines, fewer pollens	31.5^{bc}
CV%				0.6
F_{value}				11.96^{**}

In the same column, numbers with the same letter do not had a statistically significant difference; ns: no significant;*: significant difference at the level of $\alpha = 0.05$; **: very significant difference at the level of $\alpha = 0.01$.

Table 5. Characteristics of 3 popular cucumber varieties in Vietnam's Southeastern market (survey results in May, 2019)

Variety	Company	Days to flower initiation	Fruit shape	Fruit peel colour	Average weight per fruit (g)	Fruit diameter (cm)	Fruit length (cm)	Average yield (tons/ha)	Cultivation season	Advantages	Disadvantages
Hunter 2.0	Eastwest seed	32	Cylinder, long, short	Light green	140	3.5	14 - 16	30	Annunal	Strong plant, long term harvest	Short fruit
SL 1.2	An Phu Nong	32	Cylinder, long	Light green	160	3.5	16 - 17	33	Annunal	Strong plant, concentrated fruit setting	Light green fruit, weak
Vino 67	Viet Nong	34	Cylinder, long	Green	180	3.5	16 - 18	28	Annunal	Strong plant, sweet fruit	Late fruit setting, fewer fruit

than the control variety.

In the market for cucumbers, fruit sizes that are too big or too small are unpopular. Fruit sizes of some popular varieties in Vietnam's Southeastern are 16-18 cm long, with an average weight of 160-180 g per fruit. The C2 x C44; C31 x C44; C35 x C44 and C44 x C52 hybrid combinations had suitable fruit shape.

In terms of yield, the C20 x C40 hybrid combination produced the highest yield of 39.8 ton/ha and the difference was statistically significant compared to the C2 x C44, C4 x C37, C12 x C44. C35 x C44 hybrid combinations and the control variety Hunter 1.0. According to the results in Table 5, the varieties currently available in the Southeast had the average yields from 28 to 33 ton/ha and significantly lower than the C20 x C40 hybrid combination.

Regarding to fruit shape, all hybrid combinations have a cylindrical shape and conical top similar to Hunter 1.0. However, the C4 x C37, C20 x C40 and C41 x C52 hybrid combinations produced spherical bottom, which was different from the rest of the hybrid combinations and the control that had the circular bottom (Table 4). In addition, fruits from the hybrid combinations have similar shape and colour to the varieties available in the Southeast, according to the survey results in Table 5.

Next, the peel of cucumber in all treatments was green, thin, and soft white spines. In which, cucumbers of 3 hybrid combinations C2 x C44, C20 x C40 and C35 x C44 had short stripes differing from the remaining hybrids and including the control variety. In addition, the C12 x C44, C20 x C40 and C44 x C52 hybrid combinations had a medium pollen layer, and the rest of the hybrid combinations, including the control variety, had fewer pollen on the pods.

Finally, cucumbers in the C4 x C37, C12 x C44 and C44 x C52 hybrid combinations had the highest fruit hardness with values of 32.0, 32.0, 31.6 Ibf, respectively, and the difference was very statistically compared to other hybrid combinations and the control variety Hunter 1.0.

4. Conclusions

The hybrid combinations all grew and developed well under the same experimental conditions. The three C20 x C40, C31 x C44; C44 x C52 hybrid combinations had a higher yield than Hunter 1.0 control. However, the C31 x C44; C44 x C52 hybrid combinations had the average fruit weight equivalent to the control variety, and equivalent to some popular cucumber varieties in the Southeastern market, while the hybrid C20 x C40 had a smaller fruit size compared with popular varieties (as survey results mentioned in Table 5). About the morphological characteristics, the C31 x C44, C44 x C52 hybrid combinations had fruit shape and fruit colour similar to Hunter 1.0. However, the C31 x C44 hybrid combination had low hardness, leading to damage during transport and storage easily. On the other hand, the C44 x C52 hybrid combination had high hardness and similar to the control variety.

In conclusion, it is reasonable to choose the C44 x C52 hybrid combination as a promising hybrid cucumber variety for Vietnam's Southeastern market.

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Multiplex RT-PCR assay to differentiate genotypes of porcine reproductive and respiratory syndrome virus in swine

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ABSTRACT

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases to swine industry worldwide. Due to the heterogeneity of field isolates, accurate detection of the PRRS virus is a diagnostic challenge. Recently, co-infection with NA-PRRSV, EU-PRRSV and HP-PRRSV isolates continuously increases in many countries, resulting in a significant impact on PRRSV diagnostics and disease control on farms. To facilitate rapid diagnosis and reliable discrimination of NA-PRRSV, EU-PRRSV and HP-PRRSV, a multiplex RT-PCR assay was established with three pairs of primers targeting highly conservative regions of nsp2 gene with predicted multiplex RT-PCR products of 364 bp, 161 bp and 259 bp, respectively. The primer pairs were optimized to be highly specific for PRRSV genotypes and were able to detect the target gene at the limit of 10^2 copies/uL for each gene. Clinical samples were used to evaluate this multiplex RT-PCR in parallel with a commercial realtime RT-PCR kit. Results showed over 95.2% (20/21 samples) agreement between the mRT-PCR and the real-time RT-PCR kit. Hence, it indicated that this multiplex RT-PCR could be useful for rapid and differential diagnosis of NA-PRRSV, EU-PRRSV and HP-PRRSV in swine farms.

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1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) or "blue ear" disease is a highly contagious viral disease that was first recognized in North America in the late 1980s, followed in Europe and Asia. The main clinical signs of this disease are late-term reproductive failures in sows as well as reduction in rate of growth and increased mortality rate in all age groups due to severe pneumonia (Terpstra et al., 1991; Rossow et al., 1994). Thus, PRRS causes significantly economic loss in most swine-producing countries worldwide.

The etiological agent of PRRS is porcine reproductive and respiratory syndrome virus (PRRSV) which belongs to the Nidovirales order, Arterivirus genus and Arteriviridae family (Cavanagh, 1997). Porcine Reproductive and Respiratory Syndrome virus is an enveloped, single positive-stranded RNA virus with a genome ranging from 13 to 15 Kb in length, which contains at least 10 open reading frames (ORFs). The ORF1a and ORF1b comprise more than 75% of the genome and encode non-structural polyproteins responsible for viral replication (Kappes & Faaberg, 2015). The ORF2, ORF3 and ORF4 encode for the minor structural surface proteins GP2, GP3 and GP4, respectively and together these proteins form a trimeric complex that is heavily N-glycosylated and functions in viral entry (Das et al., 2010, 2011). Furthermore, ORF5,

ORF6 and ORF7 encode for the major structural surface proteins GP5, matrix (M) and nucleocapsid (N), respectively. Besides, the N-glycosylated GP5, a unglycosylated protein is translated from an alternative reading frame, designated ORF5a (Johnson et al., 2011; Kappes & Faaberg, 2015).

Phylogenetic analysis of this causative agent isolates worldwide indicates clearly existence of two major genotypes, the European genotype (EU-PRRSV or type 1) and North American genotype (NA-PRRSV or type 2) with Lelystad and VR-2332 as prototypical strains, respectively (Meng et al., 1995). Both types share an approximately 60% sequence identity at the nucleotide level (Nelsen et al., 1999). Until May 2006, a new variant of PRRSV emerged, later defined as a highly pathogenic form of PRRSV (HP-PRRSV), and severely impacted the pig industry with the death of more than two million pigs in South China (Tian et al., 2007). At the molecular level, common characteristics of all highly virulent PRRSV isolates were usually demonstrated by a unique discontinuous deletion of 30 amino acids (aa) in the non-structural protein 2 (*nsp2*) that has been proposed as the genetic marker of the HP-PRRSV (Tian et al., 2007).

The diagnosis of PRRSV has been accomplished typically by virus isolation, immunohistochemistry, serological methods, or conventional reverse transcriptase PCR (RT-PCR) (Mengeling et al., 2000). The drawbacks of these methods are time-consuming, labor-intensive, potential for cross-contamination and high costs. Obviously, RT-PCR assay is highly specific and sensitive, however it is difficult for PRRSV genotype or strains discrimination. Meanwhile, the co-infection among different PRRSV genotypes has been increasingly reported, resulting in significant challenges on PRRSV diagnostics and management. Therefore, a rapid and reliable multiplex RT-PCR assay for differential diagnosis of NA-PRRSV, EU-PRRSV and HP-PRRSV strains in this study is quite useful for routine diagnosis of PRRS in swine farms.

2. Materials and Methods

2.1. Nucleic acid for reaction controls and clinical samples

The RNA *genome* of NA-PRRSV (VR-2332, Genbank ID U87392), EU-PRRSV (DV, similar with Lelystad strain, Genbank ID M96262) and HP-PRRSV (JAX1, Genbank ID EF112445) strains used in this research were extracted from live-attenuated vaccines, includes Ingelvac PRRS MLV vaccine (Boehringer, Germany), Porcilis PRRS vaccine (Intervet, Netherland) and a vaccine product of Hoa Nong Company (China), accordingly. Viral RNA was extracted by TRIzol Plus RNA Purification kit (Cat#12183555, Invitrogen, USA).

Genetic material of other viruses and bacteria, including Classical swine fever virus (CSFV), Pseudorabies virus (PRV), *Haemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Streptococcus suis*, *Staphylococcus aureus* and DNA of pig used for evaluation of the specificity of the mRT-PCR were provided by the Sanphar Vietnam laboratory (Erber group, Austria) and IDT (United States).

Regarding clinical samples, a total of 21 serum (n = 10) and lung tissue samples (n = 11) were collected from swine farms in Dong Nai and Binh Duong provinces and were used as field sample to evaluate the potential application of the established mRT-PCR.

2.2. Primer design

Three pairs of primers distinguishing highly pathogenic PRRSV (HP) versus PRRSV type 1 (EU) and PRRSV type 2 (NA) were designed in *nsp2* gene segment by MPRIMER software. The nucleotide sequence of target gene obtained from NCBI (Table 1), validated by NCBI BLAST, OligoAnalyzer 1.0.2 software. The annealing temperature and the size of the amplified product were adjusted for clear segregation in electrophoresis. Primers were synthesized by IDT (Integrated DNA Technologies, USA).

2.3. RT-PCR optimization

Before setting up mRT-PCR, all primers were evaluated for functioning in single RT-PCRs according to the predicted annealing temperature and regular RT-PCR protocol. The sRT-PCR was performed in a 30 uL reaction containing 15 µL PCRBIO 2X One Step RT-PCR kit (Cat#PB105210, PCR Biosystems, England), 1.5 µL RTase 20X, 0.5 µM of each primer, 2 µL of RNA template, and nuclease-free water to adjust the final volume to 20 µL. Nuclease-free water was also used as a negative control for all PCRs. The RT-PCR was carried out under the

Table 1. Primers used in this study

Name	Sequences (5'-3')	size(bp)
NA364	F: CCTGGGTGTGGTGTTTCT R: ATGGCGACGTGTTAGGCT	364
HP259	F: ATCATGACCAGGTGTGCTTG R: TGTTCGGTTGTCTGATTCGC	259
EU161	F: GGTGGAATGTACTCCATCCG R: CTTGGAGTTCACGAAGGTGT	161

following conditions: a reverse transcription step at 45°C for 10 min, reverse transcriptase inactivation and pre-denaturation at 95°C for 2 min, followed by 40 cycles consisting of denaturation at 95°C for 15 sec, annealing at optimal temperature for 30 sec, and extension at 72°C for 30 sec, Ten µL of amplified products were separated on 2% agarose gel electrophoresis in 0.5X Tris-Borate-EDTA (TBE) with Midori Green Advance DNA stain (Cat#AG10, Nippon) and 1 kb Plus DNA ladder (Cat#10787018, Invitrogen) as the molecular weight markers to indicate the sizes of the amplified products. The effects of annealing temperature ranging from 52°C to 60°C were also determined experimentally.

2.4. Optimization of multiplex RT-PCR assay

To establish a functional mRT-PCR, several aspects need to be examined such as annealing temperatures, primer concentration, primer ratio,... After several rounds of optimization, the final primer mix with a ratio of 0.5 µM (NA): 0.5 µM (EU): 0.5 µM (HP) was accordingly achieved. The final multiplex RT-PCR mixtures contained 15 µL PCRBIO 2X One Step RT-PCR kit, 1.5 µL Rtase 20X, 0.5 µM of each of the three primers, 2 µL of RNA template and nuclease free water up to 20 µM in total. Thermal cycling conditions and gel electrophoresis were similar to the one described above for sRT-PCR.

2.5. Specificity and detection limit

To evaluate the specificity of the mRT-PCR assay, DNA of different viruses and bacteria that are commonly found in swine farms and potentially contaminate during sampling process were used as unrelated template in reactions. For confirmation of the mRT-PCR products, DNA bands were recovered from low melting agarose gel (Cat#BP165-25, Thermo Fisher Scientific, USA), using phenol-chloroform method (Green & Sambrook, 2012), and sequenced by Nam Khoa Biotek company. Later on, the resultant nucleotide sequences of the mRT-PCR products were aligned with reference sequences in Genbank using BLAST tool to verify the specificity of the mRT-PCR.

Concerning the detection limit, the purified RNA templates were first quantified by Realtime PCR (PowerCheckTM PRRSV Realtime PCR, Kogenebiotech, Korea) then were diluted 10-fold serially in nuclease-free water. The diluted template were further used to determine the detection limit of the established mRT-PCR.

2.6. Application in diagnosis of clinical samples

The potential application of the established mRT-PCR assay in routine diagnosis of PRRSV for field samples was determined by head to head comparison with the results produced by commercial Realtime PCR assay. A total of 21 clinical samples were analyzed in this study.

3. Results and Discussion

3.1. mRT-PCR establishment

In sRT-PCR reactions to examine the general conditions for the assay, gel electrophoresis analysis showed the correct product sizes as predicted, including 364 bp for NA PRRSV, 259 bp for HP PRRSV, and 161 bp for EU PRRSV. The results also indicated that 3 pairs of primers worked properly in the annealing temperature range of 52°C – 60°C (data not shown), and the 54°C was chosen for mRT-PCR in this study. The primer concentration and ratio were 1 NA : 1 HP : 1 EU at 0.5 µM per reaction. Additionally, the three products were clearly visible and distinguishable from each other, no visible band was found in the lane of negative control (Figure 1).

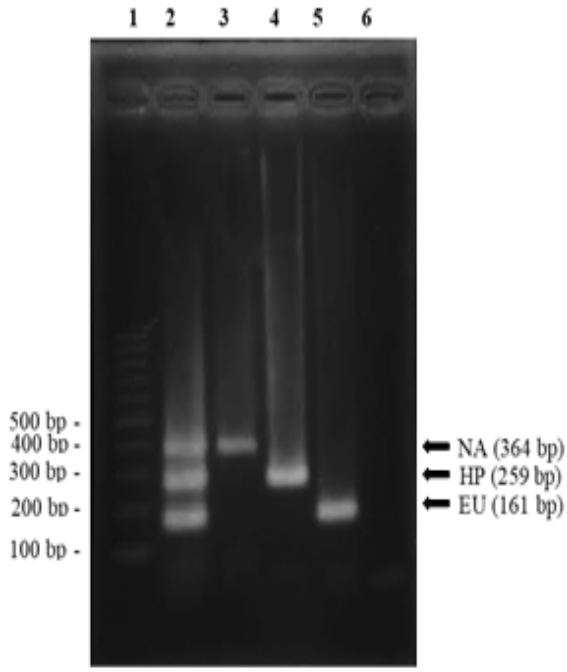

Figure 1. Products of sPCRs and mPCR. (1): DNA ladder 1 Kb plus, (2): mRT-PCR of all three targets, (3): NA-PRRSV 364 bp, (4): HP-PRRSV 259 bp, (5): EU-PRRSV 161 bp, (6): negative control with pure water. The thermal cycling conditions were 94°C/5 min; 35 cycles of 94°C/30 sec, 54°C/30 sec and 72°C/40 sec, a final extension at 72°C/5 min; Gel electrophoresis was 2% agarose and performed at 90 vol/25 min.

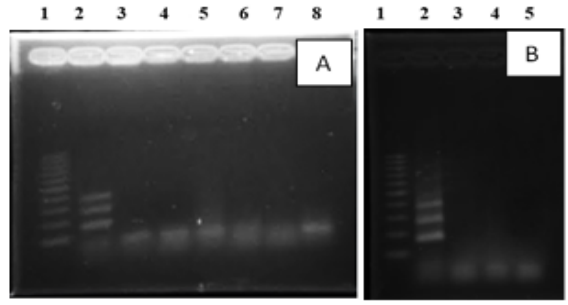

Figure 2. Specificity of mRT-PCR. A. (1): ladder 1 Kb plus, (2): positive control, (3): negative control with pure water, (4): *Streptococcus suis*, (5): *Staphylococcus aureus*, (6): *Mycoplasma hyopneumoniae*, (7): Pseudorabies virus, (8): Classical swine fever virus. B. (1): ladder 1 Kb plus, (2): positive control, (3): pig DNA, (4): *Haemophilus parasuis*, (5): negative control with pure water. The thermal cycling conditions were: 94°C/5 min; 35 cycles of 94°C/30 s, 54°C/30 s and 72°C/40 s, a final extension at 72°C/5 min; Gel electrophoresis was at 90 vol/25 min.

3.2. Specificity and detection limit of the mRT-PCR

After the primer ratio and the thermal cycling conditions have been established. The specificity and the detection limit of the mRT-PCR were determined. Based on the routine usage of blood or serum in practical disease monitoring which can also contain different microbes due to septicemia or contamination, unrelated nucleic acid templates isolated from bacteria and/or viruses commonly found in pig farms were used in this experiment, including *Staphylococcus aureus*, *Streptococcus suis*, *Mycoplasma hyopneumoniae*, Pseudorabies virus, Classical swine fever virus and genomic DNA from pigs. Results showed that the three primer pairs did not cross-react with any of these templates (Figure 2), indicating the established mRT-PCR has expected specificity. We then evaluated the limit of detection of the mRT-PCR by performing the assay with a set of serially 10-fold diluted positive control templates with the copy numbers ranging from 2 x 10^3 to 2 x 10^0 copies per reaction. As shown in Figure 3, three distinct DNA bands at expected size were observed at the concentration of 2 x 10^2 copies per reaction. It demonstrated that this mRT-PCR could detect the viral genes at the limit of 200 copies/gene/reaction.

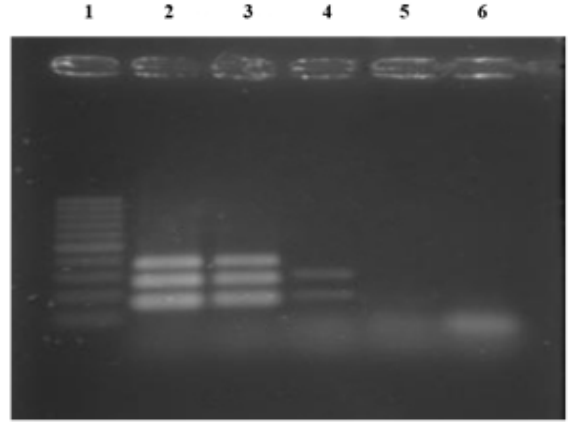

Figure 3. Detection limit of mRT-PCR. (1): ladder 1 Kb plus, (2-5): RNA template of NA PRRSV, HP PRRSV and EU PRRSV at 2 × 10^3 - 2 × 10^0 copies/reaction, (6): negative control with pure water. The thermal cycling conditions were: 94°C/5 min; 35 cycles of 94°C/30 sec, 54°C/30 sec and 72°C/40 sec, a final extension at 72°C/5 min; Gel electrophoresis was at 90 vol/25 min.

Table 2. Detection of clinical specimens by multiplex RT-PCR and Realtime RT-PCR

Pig farm	No. of specimens	mRT-PCR			Realtime RT-PCR		
		NA	HP	EU	NA	HP	EU
1	1.1	+	+	-	+	+	-
	1.2	-	+	-	-	+	-
	1.3	-	+	+	-	+	+
2	2.1	+	+	-	+	+	-
	2.2	+	-	-	+	-	-
	2.3	+	-	-	+	-	-
3	3.1	-	+	-	-	+	-
	3.2	+	+	-	+	+	-
	3.3	-	-	+	-	-	+
4	4.1	+	+	-	+	+	-
	4.2	-	+	-	-	+	-
	4.3	-	-	+	-	-	+
5	5.1	+	-	-	+	-	-
	5.2	-	+	+	-	+	+
	5.3	+	-	-	+	-	-
6	6.1	-	+	-	+	+	-
	6.2	+	+	-	+	+	-
	6.3	+	-	-	+	-	-
7	7.1	-	+	+	-	+	+
	7.2	-	-	+	-	-	+
	7.3	-	+	-	-	+	-
Total of positive samples		10	13	6	11	13	6
Concordance rate %		90.9 (10/11)	100 (13/13)	100 (6/6)			

This detection limit is comparable to the result reported by Xiao et al. (2014). In that study, the author used RT-PCR developed based on SYBR Green and TaqMan probe to discriminate highly pathogenic PRRSV against NA PRRSV, result of detection limit was 5×10^2 copies/ reaction. In addition, Balka et al. (2009) reported the detection limit in their Realtime RT-PCR assay to be approximately 10 copies/reaction. Other research provided that mRT-PCR for detection EU PRRSV, HP-PRRSV and NA PRRSV has an analytical sensitivity of less than 200 copies/reaction (Wernike et al., 2012). Therefore, detection limit of mRT-PCR achieved in this study was quite suitable for routine diagnosis.

3.3. Detecting the presence of PRRS virus from clinical samples

To examine the utility of the mRT-PCR for routine diagnosis of PRRSV genotypes circulating in swine farms which can help to guide the farm in choosing appropriate vaccines. A total of 21 samples including serum and tissues were used in parallel testing between the mRT-PCR and a commercial Realtime RT-PCR kit. Result summarized in Table 2 showed that the agreement between the mRT-PCR and realtime RT-PCR assays was 90.9% (10/11 samples), 100% (13/13 samples) and 100% (6/6 samples), respectively for NA-PRRSV, EU-PRRSV and HP-PRRSV. Interestingly, co-infection among PRRSV strains were observed in these field samples. In details, there were 3 samples positive for both HP-PRRSV and EU-PRRSV, 6 samples positive for both HP-PRRSV and NA-PRRSV. It indicated that the mRT-PCR would be useful for differential diagnosis and/or determination of co-infection of various genotypes in a swine herd.

4. Conclusions

In summary, the established mRT-PCR was a convenient, rapid, sensitive and specific assay for the identification of different PRRSV genotypes. The size of amplified products is distinguishable. The method showed a feasible application in regular laboratory testing for viral surveillance

and for effective control of the NA-PPRSV, EU-PRRSV and HP-PRRSV strains in swine farms.

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Influence of stocking density on water quality and growth performance of whiteleg shrimp (*Litopenaeus vannamei*) reared in fiberglass tanks, without water exchange

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ABSTRACT

The present study evaluated the effects of stocking density on water quality parameters, growth performance and survival rate of white leg shrimp *Litopenaeus vannamei*, reared in fiberglass tanks, without water exchange. Three stocking densities (50, 100 and 200 shrimp/m^2) were tested. Each treatment consisted of three replicate fiberglass tanks (500 L). The shrimp were fed ad libitum four times per day with a commercial pellet (40-42% protein). After an 8-week trial, concentrations of nutrients in the culture tanks showed an increasing linear relationship with increasing stocking density. The growth performance of shrimp in low stocking densities was significantly greater than that in high stocking densities. The results from this study demonstrate that with increasing the stocking density the production of shrimp increased but in a low final weight and survival compared to low stocking density.

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1. Introduction

In Vietnam, since the 1990s, shrimp has become an important brackish species in farming and the main product of seafood export. The area under farming was increased annually at a high growth rate. Addition to increased farming area, the farm production of shrimp in Viet Nam was also diversified in cultured systems and intensified in stocking density, inputs and management (Nguyen et al., 2019). Although shrimp farming has contributed significantly to the socio-economic development of the country, it has increased in pollution of land and water and resulted in the degradation of the environment.

One of the major environmental concerns relate to intensive aquaculture is the discharge of nutrient-rich effluents into the water bodies. During the shrimp farming cycle, solid wastes (uneaten food and feces) settled at the pond bottom and negatively impact on the water quality. The nutrient retention by shrimp ranged from between 6% and 30% for carbon (C), 17% and 45% for nitrogen (N), and 6.1% and 21.3% for phosphorus (P), whereas most of the N (24 – 49%), P (24 – 84%), and C (65%) not recovered in harvested shrimp accumulated in the culture system (Avnimelech & Ritvo, 2003; Sahu et al., 2013). Boopathy et al. (2007) reported that concentrations of chemical oxygen demand, total solid and total ammonia nitrogen in shrimp wastewater sludge might reach 1,201 mg/L, 13,100 mg/L and 102 mg/L, respectively, exceeding the limits of wastewater discharge standard.

The density-dependence in growth and mortality in shrimp were reported in several works (Allan & Maguire, 1992; Moss & Moss, 2004; Legarda et al., 2018). An increase in stocking density from 5 to 40 shrimp/m^2 had no effect on the survival of *Penaeus monodon* and water quality, but growth declined and yield increased as density increased (Allan & Maguire, 1992). Similarly, as the stocking density of shrimp *P. stylirostris* increased from 1 to 30 shrimp/m^2, the shrimp weight and survival rate decreased significantly and the amount of wastes produced was relative to the stocking density (Martin et al., 1998). Yu et al. (2009) reported that increasing stocking densities reduced the growth performance and survival rate of cultured shrimp and water quality deterioration. Similarly, Arnold et al. (2009) and Arambul-Muñoz et al. (2019) found that density-dependent growth and survival in intensive shrimp culture could be mainly due to a combination of factors, including a decrease in the availability of natural food sources and space, an increase in cannibalism, decrease in water quality and accumulation of undesirable sediments.

The main objective of the farmers in Viet Nam is to maximize profit per unit area, usually leading to more environmental problems. However, the high density of shrimp may be far over the carrying capacity of the pond. An inverse relationship between stocking density and shrimp growth in Viet Nam was reported (Nguyen et al., 2013), but there has been limited research on this topic, particularly at a density higher than 100 shrimp/m^2. Therefore, this study was carried out to assess the effects of three stocking densities of whiteleg shrimp (*Litopenaeus vannamei*) on (i) water quality, and (ii) growth performance and feed utilization of shrimp.

2. Materials and Methods

2.1. Shrimp, seawater, and feed

This study was performed at the experiment farm, Faculty of Fisheries, Nong Lam University, Ho Chi Minh City, Vietnam. Juvenile whiteleg shrimp *L. vannamei* (0.82 ± 0.07 g) were purchased from a private hatchery nearby the university. Shrimp were transported in 45-L styrofoam box equipped with air pumps to the experiment farm. Shrimp were acclimatized in 1 m^3-fiberglass tank to the experimental condition for one weeks and fed commercial pellets thrice per day.

The culture water salinity of 15‰ in the trial was made by diluting natural seawater with tap water, disinfected by chlorine of 30 ppm for 3-4 days and neutralized with sodium thiosulfate to remove chlorine residue before use.

Three size classes of commercial feed produced by C.P. Viet Nam Corporation (Bien Hoa, Dong Nai, Viet Nam) were utilized according to different shrimp's growth stages.

2.2. Experimental procedure and management

The experimental design was completely randomized with three treatments (three shrimp densities): 50, 100 and 200 shrimp/m^2 (abbreviation of D50, D100 and D200, respectively), each treatment repeated 3 times. The experiment was conducted in nine 500 L-fiberglass tanks, coupled with a biofilter, with a water volume maintaining at 400 L. The tank was equipped with an air-stone and two 100-W aquarium thermostat heaters.

Every tank had an associated, 10 L biofilter (ϕ26.4 x 24.6 cm), made from a plastic bucket. Biofilter design using downflow trickling filter was placed above the shrimp tank. Water went into the biofilter by way of a 27 mm airlift pipe, moving from the base of the tank. Water from the airlift passed through a fine mesh on the top of the biofilter, then flowed over the crushed coral media (media depth of 18 cm) and out an array of 1 cm holes (drilled in the bottom) and back into the shrimp tank. During the experiment, water was recirculated with no water exchange, the only freshwater added was that needed to compensate for evaporation and sampling.

The experiment lasted for 8 weeks. During the experiment, the shrimp were hand-fed to apparent satiation four times per day (at 7:00, 12:00, 17:00 and 22:00 h) using circular feeding trays (ϕ40 cm). One hour after feeding, uneaten feed and feces were removed. To estimate the feed intake, all uneaten feed found in feeding trays was collected, dried in the oven, and weighted.

2.3. Water sampling and analyzing

Water quality variables such as temperature, pH, and dissolved oxygen (DO) were measured twice daily (07:00 – 08:00 and 16:00 – 17:00) by HP3040 pH/mV/Temp. Meter and Milwaukee MW 600 DO Meter, respectively. Routine

bi-weekly water sampling was done at 07:00 from each tank, kept in an ice box, transferred to the laboratory of Faculty of Fisheries, Nong Lam University within 2 h and analyzed within 24 h. Water quality parameters, e.g., alkalinity, total suspended solids (TSS), nitrogen compounds (ammonia (TAN), nitrite (NO_2-N), nitrate (NO_3-N), total Kjeldahl nitrogen (TKN)), phosphorus (total and soluble reactive phosphorus (TP and SRP)) and total organic carbon (TOC) were analyzed following the standard methods for water and wastewater analysis (APHA, 2012). Alkalinity and TSS were determined by titration method (2320 B) and filtered and dried at 103-105°C (2540 D), respectively. Levels of TAN, NO_2-N, NO_3-N and TKN were analyzed by phenate method (4500-NH_3 F), colorimetric method (4500-NO_2- B), cadmium reduction method (4500-NO_3- E) and macro-Kjeldahl method (4500-Norg B), respectively. While levels of TP digested by alkaline potassium persulfate and SRP were determined by ascorbic acid colorimetric method (4500-P E), respectively. Water samples for TOC were kept frozen at -18 ± 2°C, transported to the Advanced Technology Laboratory, Can Tho University, Can Tho City, Viet Nam and analyzed by LAR's TOC analyzer.

2.4. Calculations and statistical analysis

Weekly weight gains (WG), specific growth rate (SGR), feed conversion ratio (FCR) and survival rate (SR) were evaluated as follows:

$$\text{Weekly weight gain (WG,g/day)} = \frac{W_f - W_i}{\Delta t}$$

$$\text{Specific growth rate (SGR,\%/day)} = \frac{\ln W_f - \ln W_i}{\Delta t} \times 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed consumed (dry matter)}}{\text{wet weight gain}}$$

$$\text{Survival rate (SR,\%)} = \frac{\text{final number of shrimp}}{\text{initial number of shrimp}} \times 100$$

where W_f and W_i represent the final and initial body weights of shrimp individuals in each tank, respectively (g); and Δt is the duration of the experiment (8 weeks, equal to 56 days).

Survival rates were arcsine square root transformed but untransformed values are shown here. Water quality parameters were compared using one-way repeated measure ANOVA with treatments (stocking density) as the main factor and time as repeated measures (Gomez & Gomez, 1984). Bonferroni test was used when differences between time and treatments were detected ($P < 0.05$). Data obtained from shrimp performance (WG, SGR, FCR and SR) were analysed using one-way ANOVA to determine the effect of stocking densities. Duncan multiple range test (Duncan test) at $P < 0.05$ level of significance was employed to compare mean differences between treatments. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 19.0 (Armonk, NY: IBM Corp). All data were expressed as the mean ± standard deviation (SD).

3. Results and Discussion

3.1. Parameters of water quality

Water temperature, pH and DO levels in all tanks tended to be relatively stable (Table 1) and were maintained within acceptable limits for the maximum growth of *L. vannamei* (Wyk & Scarpa, 1999; MARD, 2014).

The water quality parameters monitored bi-weekly are given in Table 2. No significant differences ($P > 0.05$) between treatments were found in water quality regarding TSS, alkalinity and TAN. However, significant differences ($P < 0.05$) were observed for nitrite, nitrate, TKN, SRP, TP and TOC among the treatments (Table 2). In general, levels of these parameters at a density of 50 shrimp/m^2 showed the lowest, while the treatment 200 shrimp/m^2 provided the highest, followed by the treatment 100 con/m^2.

The results showed that TSS concentrations were relatively low and increased significantly ($P < 0.05$) over time, on average, ranging from 1.76 mg/L at stocking to 10.2 mg/L at harvest (Figure 1). Alkalinity decreased gradually in all treatments, particularly at high stocking density, though regular adding sodium bicarbonate to maintain alkalinity (Figure 1). The recorded low alkalinity values were due to the consumption of inorganic carbon by the nitrifying bacteria (Ebeling et al., 2006). According to Chen et al. (2006), for every gram of TAN being oxidized to nitrate, about 7.07 g of alkalinity is consumed.

Concentrations of TAN fluctuated significantly ($P < 0.05$) between sampling times, and the high-

Table 1. Water temperature, pH and DO levels (mean ± SD) of all tanks with different stocking densities

Parameters	Time	Treatment		
		D50	D100	D200
Temperature (°C)	Morning	29.4 ± 1.0	29.8 ± 0.8	29.3 ± 1.0
	Afternoon	29.8 ± 0.8	29.4 ± 1.0	29.4 ± 0.9
DO (mg/L)	Morning	6.52 ± 0.27	6.38 ± 0.33	6.26 ± 0.35
	Afternoon	6.38 ± 0.33	6.35 ± 0.31	6.16 ± 0.33
pH	Morning	7.72 ± 0.44	7.71 ± 0.10	7.61 ± 0.12
	Afternoon	7.71 ± 0.10	7.67 ± 0.11	7.65 ± 0.10

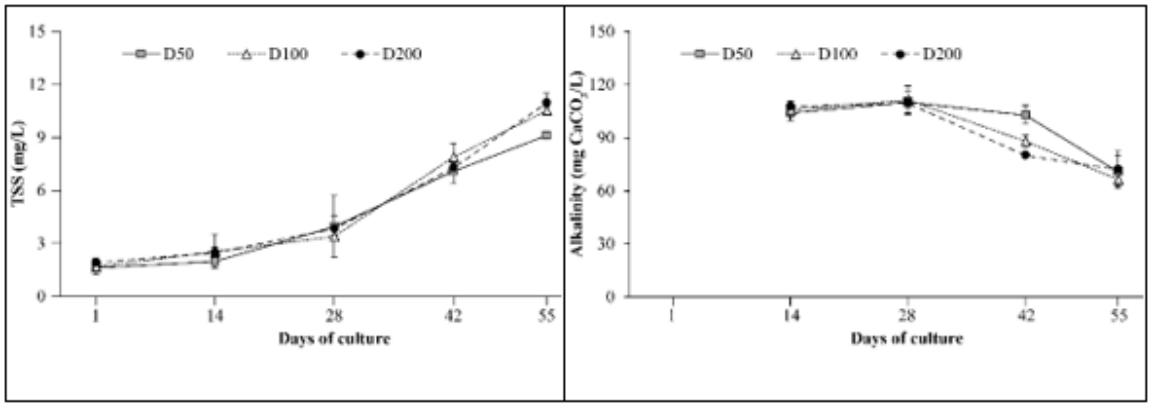

Figure 1. Effects of stocking densities on variations of levels of TSS and alkalinity in the culture of *L. vannamei* during the study. Values are means and standard deviations at sampling times (n = 3).

Table 2. Effects of stocking densities on the water quality parameters (mg/L, except alkalinity as mg $CaCO_3$/L) during the study

Parameters	Treatment			Significance (P values)‡		
	D50	D100	D200	Density	Time	Density × Time
TSS	4.75 ± 3.10†a	5.21 ± 3.60a	5.29 ± 3.51a	ns	< 0.001	< 0.01
Alkalinity	96.9 ± 17.0a	92.9 ± 18.9a	92.3 ± 17.9a	ns	< 0.001	< 0.05
TAN	0.96 ± 0.58a	1.20 ± 0.56a	1.57 ± 0.95a	ns	< 0.001	ns
NO_2^--N	0.26 ± 0.32a	0.53 ± 0.58b	0.80 ± 0.87c	< 0.01	< 0.001	< 0.01
NO_3^--N	16.6 ± 5.8a	28.1 ± 12.2b	40.7 ± 23.1c	< 0.001	< 0.001	< 0.001
TKN	2.53 ± 1.93a	4.29 ± 3.05b	5.16 ± 3.65b	< 0.01	< 0.001	< 0.001
SRP	1.62 ± 0.85a	1.91 ± 0.93b	2.39 ± 1.11c	< 0.001	< 0.001	< 0.001
TP	4.81 ± 3.05a	6.11 ± 4.17b	7.84 ± 5.85c	< 0.01	< 0.001	< 0.001
TOC	14.2 ± 5.3a	20.0 ± 7.2b	22.1 ± 8.1b	< 0.05	< 0.01	ns

†Values represent the mean ± SD (n= 15, 3 replicate × 5 sampling time).
Mean values in the same row with different superscript letters differ significantly (Bonferroni test, $P < 0.05$).
‡Results from one-way repeated measure ANOVA and Bonferroni test: Density = stocking densities; Time = sampling time and Density × Time = interaction between stocking densities and sampling time. ns: not significant ($P > 0.05$).

est peak of TAN concentrations of all treatments was observed in the treatment D200 on week 6 of the culture period (3.7 mg/L) (Figure 2). Using the calculation suggested by Boyd & Tucker (1998), toxic ammonia (NH_3) reached a maximum value of 0.185 mg/L (at highest pH of 7.8 and temperature of 30°C), while the lethal concentration (LC_{50}) of NH_3 to *L. vannamei* is 2.78 mg/L (Lin & Chen, 2001). Also, this concentration was below the 0.3 mg/L critical value for shrimp set by QCVN 02-19: 2014/BNNPTNT (MARD, 2014).

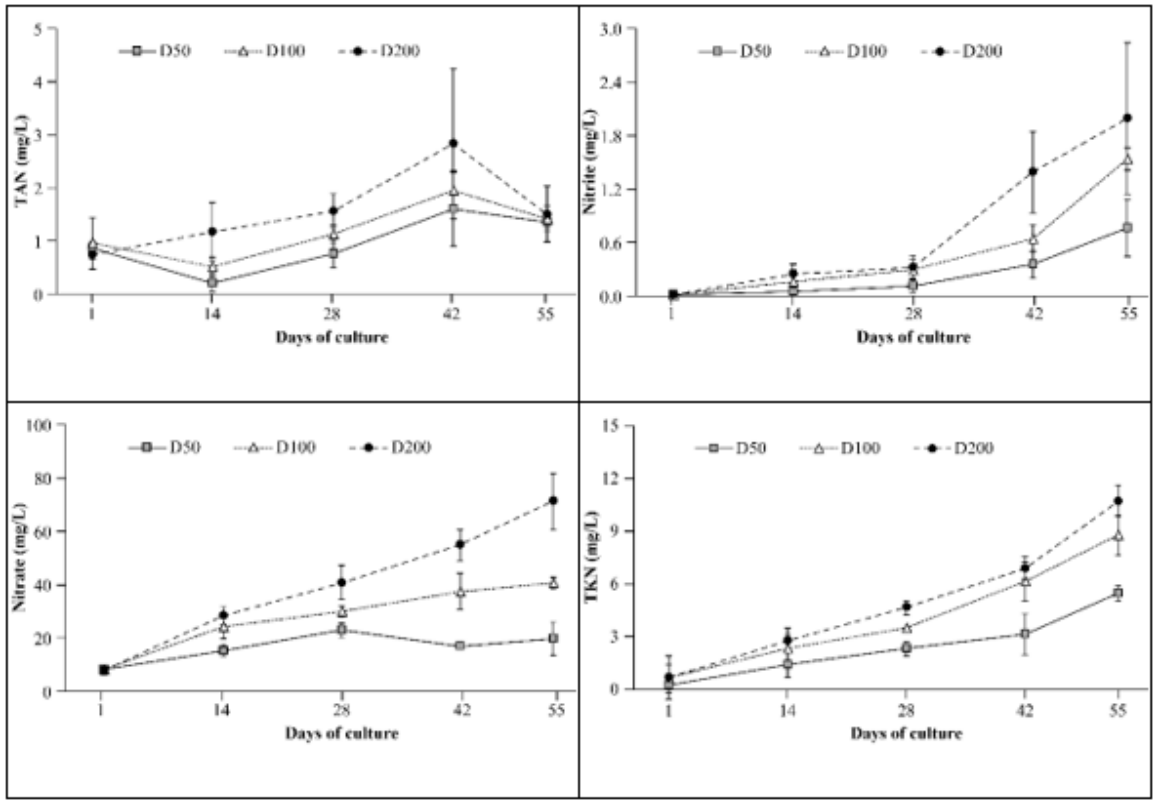

Figure 2. Effects of stocking densities on variations of concentrations of TAN, TKN, nitrite, and nitrate in the culture of *L. vannamei* during the study. Values are means and standard deviations at sampling times (n = 3).

Concentrations of nitrite and nitrate significantly increased with increasing shrimp stocking density ($P < 0.05$) (Table 2). Moreover, in all the treatments, there has been a sharp rise in nitrite and nitrate levels with time, reaching a peak at the end of the trial. At the end of the experiment, the highest NO_2-N concentration was recorded in the treatment D200 (2.00 ± 0.85 mg/L), followed by D100 (1.54 ± 0.12 mg/L) and then D50 (0.77 ± 0.32 mg/L) ($P < 0.05$) (Figure 2). The high NO_2-N concentration during the last week in the treatments D100 and D200 is lower the safe level (6.1 mg/L at 15‰ salinity and a pH of 8.0) recommended by Lin & Chen (2003). In addition, the result showed that stocking densities had significantly affected on NO_3-N levels (Table 2 and Figure 2) and NO_3-N levels tended to increase over time (Table 2). The maximum nitrate concentration in the experiment, 81 mg/L, exceeded the recommended target value for shrimp, 60 mg/L (Wyk & Scarpa, 1999). However, several studies suggested that nitrate toxicity increases with a decrease in salinity (Furtado et al., 2015; Neto et al., 2019; Valencia-Castañeda et al., 2019). Kuhn et al. (2011) demonstrated that whiteleg shrimp can be reared at a salinity of 11‰ with 220 mg/L of nitrate for 6 weeks. According to Furtado et al. (2015), nitrate concentrations up to 177 mg/L are suitable for the rearing of *L. vannamei* in systems with bioflocs, without renewal of water, at a salinity of 23. Neto et al. (2019) recommended the safety level of nitrate for *L. vannamei* cultivation is 60.05 and 127.61 mg/L of nitrate for salinities 5‰ and 10‰, respectively.

The large decrease in TAN and the large increase in the concentration of nitrite and nitrate during the latter part of the study (Figure 2) might be related to an increase in the efficiency of the biofilters.

The concentration of TKN increased consistently in all treatments and was highest at the

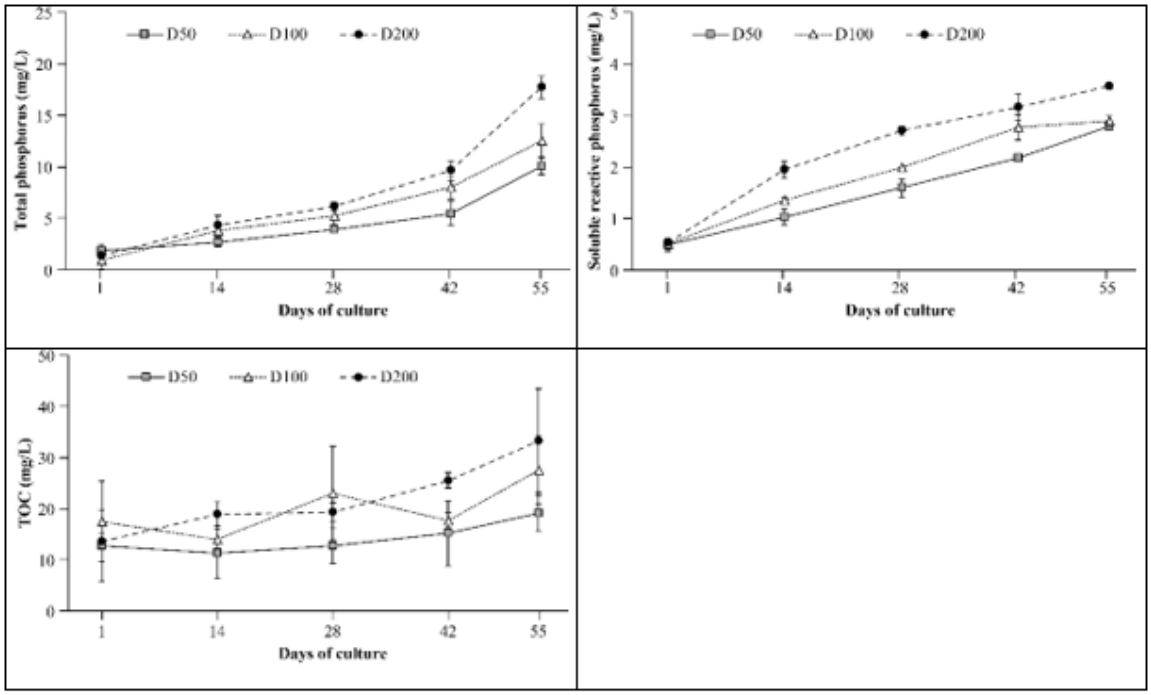

Figure 3. Effects of stocking densities on variations of TP, SRP and TOC concentrations in the culture of *L. vannamei* during the study. Values are means and standard deviations at sampling times (n = 3).

end of the study; treatment D200 had the highest TKN level (5.16 ± 3.65 mg/L), followed by D100 (4.29 ± 3.05 mg/L) and D50 was lowest (2.53 ± 1.93 mg/L). There were significant differences in TKN among treatments (Table 2 and Figure 2). An increase in TKN level could be due to the continuous supply of protein-enriched shrimp feed and no use of nitrogen removal tools such as siphon and water exchange.

Differences in concentrations of TP and SRP between treatments were statistically significant ($P < 0.05$). The treatment D200 had the highest levels of TP and SRP (7.84 ± 5.85 mg/L and 2.39 ± 1.11 mg/L, respectively), followed by D100 (6.11 ± 4.17 mg/L and 1.91 ± 0.93 mg/L, respectively) and D50 was lowest (4.81 ± 3.05 mg/L and 1.62 ± 0.85 mg/L, respectively) (Table 2). During this 8-week period, TP and SRP concentrations showed a steady increase over time among treatments (Figure 3).

Similarly, differences in concentrations of TOC between treatments were statistically significant ($P < 0.05$). The average level for TOC showed the highest in the treatment D200 (22.1 ± 8.1 mg/L), followed by D100 (20.0 ± 7.2 mg/L) and D50 (14.2 ± 5.3 mg/L) (Table 2). The TOC concentration increased throughout the experiment and reached 33.3, 27.5 and 19.1 mg/L in treatments D200, D100 and D50, respectively (Figure 3).

3.2. Shrimp growth performance and feed utilization

The results of the effect of stocking density on growth performance and feed utilization of whiteleg shrimp at three stocking densities are exhibited in Table 3. Average individual final body weight was reduced ($P < 0.05$) as stocking density increased (Table 3). The biweekly body weight of shrimp under three stocking densities is shown in Figure 4. At the end of the trial, the average final body weights of shrimp were 7.05, 6.83 and 6.24 g/shrimp at a density of 50, 100 and 200 shrimp/m^2, respectively.

Significant differences for WG and SGR were found among the density treatments ($P < 0.05$). The WG of shrimp were 0.777, 0.771 and 0.766 g/shrimp/week at 50, 100 and 200 shrimp/m^2, respectively, with significant differences ($P <$

Table 3. Effects of stocking densities on the shrimp performance of *L. vannamei* during the study

Parameters	Treatment		
	D50	D100	D200
Initial body weight (g/shrimp)	0.83 ± 0.02^{a†}	0.84 ± 0.02^{a}	0.84 ± 0.02^{a}
Final body weight (g/shrimp)	7.05 ± 0.20^{a}	6.83 ± 0.34^{ab}	6.24 ± 0.27^{bc}
WG (g/week)	0.777 ± 0.026^{a}	0.771 ± 0.03^{ab}	0.766 ± 0.022^{bc}
SGR (%/day)	3.81 ± 0.07^{a}	3.79 ± 0.07^{ab}	3.76 ± 0.02^{bc}
Survival rate (%)	80.0 ± 7.2^{a}	70.3 ± 5.5^{ab}	61.0 ± 7.8^{bc}
FCR	1.58 ± 0.12^{a}	1.72 ± 0.12^{ab}	1.96 ± 0.13^{bc}

† Values represent the mean ± SD (n= 3).
Mean values in the same row with different superscript letters differ significantly (results from one-way measure ANOVA, Duncan test, $P < 0.05$).

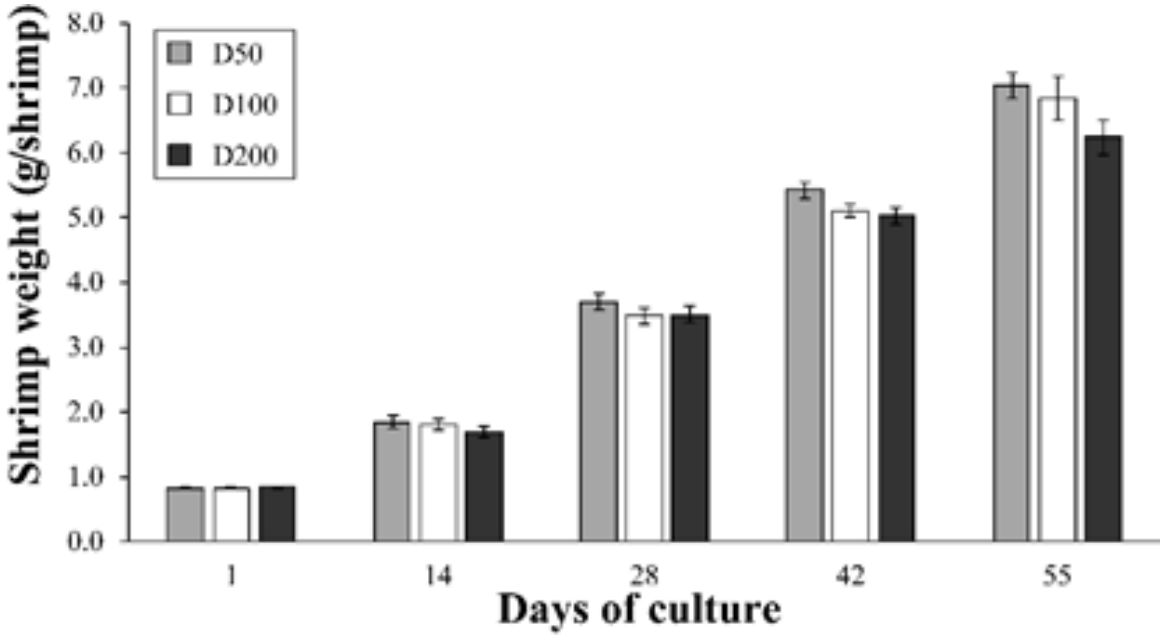

Figure 4. Effects of stocking densities on shrimp performance in the culture of *L. vannamei* during the study. Values are means and standard deviations at sampling times (n = 3).

0.05) between stocking densities. Average values of SGR were 3.81, 3.79 and 3.76 %/day at 50, 100 and 200 shrimp/m^2, respectively, with significant differences among stocking densities ($P < 0.05$).

These data indicated that growth performance was declined by increasing the density of shrimp. These results were similar to those mentioned by several authors, who found that growth in *L. vannamei* and other penaeid species is stocking density–dependent (Moss & Moss, 2004; Yu et al., 2009; Arambul-Muñoz et al., 2019). The growth performance of shrimp in this study was lower than that recorded by other authors, who found that, at stocking densities ranging of 17 – 45 shrimp/m^2, and 40 – 80 shrimp/m^2, *L. vannamei* juvenile can grow between 20.70 to 25.25 g/shrimp, and 12.4 and 16.5 g/shrimp, respectively (Sookying et al., 2011; Nguyen et al., 2013). However, in these works, the density – dependent growth was comparable, although there were differences in shrimp growth. These variations might be due to factors like environmental conditions, origin, size of organisms, experimental unit size, study periods, and culture systems.

In this work, survival rates of the whiteleg shrimp were 80.0, 70.3 and 61.0% at 50, 100 and 200 shrimp/m^2, respectively (Table 3). Values of survival rates decreased significantly (P

< 0.05) with increasing shrimp density. However, the differences between the density of 50 and 100 shrimp/m^2, and so among 100 and 200 shrimp/m^2 were not significant ($P > 0.05$). These values of survival rates are economically, technically good, acceptable and agree with those observed by Neal et al. (2010) for zero-exchange mixed biofloc systems with a survival rate of 61.8 - 89.8%. Nguyen et al. (2013) found that increasing density of whiteleg shrimp from 40 to 80 shrimp/m^2 caused in negligible but significant ($P < 0.05$) declines in survival. On the other hand, Sookying et al. (2011) found that shrimp survival was high ($> 93.4\%$) and unaffected by increasing stocking density of *L. vannamei* from 17 to 45 shrimp/m^2. The same trend was obtained by Allan & Maguire (1992) using shrimp densities from 5 to 40 shrimp/m^2 of *P. monodon*.

The average values of FCR were 1.58, 1.72 and 1.96 at 50, 100 and 200 shrimp/m^2, respectively (Table 3). Values of FCR significantly increased ($P < 0.05$) with increased shrimp density. The results of this study agreed with the previous publications under the same conditions. There was a negative correlation between stocking density and FCR with FCR ranging from 1.15 – 2.77 for *L. vannamei* and other penaeid species (Martin et al., 1998; Zaki et al., 2004; Sookying et al., 2011).

4. Conclusions

Under the experimental conditions of this work, as stocking density increased from 50 to 200 shrimp/m^2, certain parameters of water quality worsened almost linearly. Moreover, there was an obvious density-dependent relationship that revealed a decline in growth rate (final body weight, WG, and SGR), survival and increase in FCR as stocking density increased. From the abovementioned results, it could be concluded that with increasing the stocking density the production of shrimp increased but in a low final weight and survival compared to low stocking density. This problem also highlights the economic risks related to increasing stocking densities.

Conflict of interest declaration

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript.

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Production of seaweed snack from pangasius (*Pangasius hypophthalmus*) fillet by-products

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ABSTRACT

By-products from pangasius fillet processing are a good source to create a new value-added product such as snack. In this study, the effects of seaweed ratio, seasoning ratio, drying and frying conditions on sensory, texture, water holding capacity and moisture of product were investigated to find the best quality of the product.

The results showed that seaweed-added products had a better texture as compared to seaweed non-added products. However, seaweed addition > 3% weakened the product's texture. The appropriate seaweed ratio was 1%. The spice ratio (0.2% salt : 0.3% sugar : 0.1% monosodium glutamate (MSG) : 0.1% chili powder) was most favoured. The product (size 30 × 30 mm and thickness of 2 mm) was dried at 80°C in 105 min, to the final moisture of 8.17% ± 0.04. The product was fried by shortening for 20 sec at 180°C and resulted in the highest sensory quality.

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1. Introduction

In Vietnam, more than 1,000,000 tons of pangasius by-products (*Pangasius hypophthalmus*) need to be handled each year (VASEP, 2019). In particular, the source of pangasius by-products is stable, high quality and suitable to create value-added products.

One of the requirements in the production of pangasius snacks mixed with seaweed is the texture of the raw material after grinding to create gel (paste), this process is important to create products with the best texture. The gelling capacity of food proteins is an important functional attribute for food manufacturing (Zayas, 1997). Protein gelation determines the organoleptic properties of food and its texture, which, in turn, determines whether a product is accepted by the consumer (Mulvihill & Kinsella, 1987). Gelation provides texture to the food, improves water absorption, induces thickening effects, stabilizes the system and determines the appearance of food (Kinsella et al., 1985; Damodaran, 1994).

A gel is an intermediate phase between a solid and a liquid. Technically, it is defined as a substantially diluted system that exhibits no steady flow. Protein gelation refers to the transformation of protein from the sol state (viscous material) to a gel-like state (elastic material) (Haug & Draget, 2011). A gel is a three-dimensional structure that engages and immobilizes the liquid phase inside itself. Gels are obtained when pro-

teins are denatured (Mulvihill & Kinsella, 1987; Fligner & Mangino, 1991; Pilosof, 2000). Gelation is a phenomenon that occurs via crosslinking of polymers using chemical interactions. Therefore, the formed three-dimensional structures, which can trap water and low-molecular-weight substances, are called gels (Mulvihill & Kinsella, 1987; Fligner & Mangino, 1991; Pilosof, 2000). A simple definition could be that protein gelation is an aggregation of denatured molecules with a certain degree of order, resulting in the formation of a continuous network (Wong, 1989). Gelation is a two-step process: denaturation and aggregation (Kinsella et al., 1985; Matsumura & Mori, 1996). The mechanism by which proteins form a gel is not well understood. However, two theories are proposed. First, the Flory–Stockmayer theory suggests that a gelation is a sudden event that is achieved via some degree of crosslinking between polymers and reaches a critical value called the gel point, in which the viscosity diverges to infinity. Second, the Percolation theory assumes that monomers form small aggregates and, at some point, reach the gel point (Pilosof, 2000).

Seaweeds are major coastal resources that are valuable to human consumption and the environment in many countries. Edible seaweeds were widely exploited and consumed in Vietnam with the production of 600,000 – 700,000 tons of dried seaweed/year (Hoang, 2016). Thus, it is a huge demand to develop new products from seaweed. Seaweed has very little fat, ranging from 1-5% of dry matter, although seaweed lipids have a higher proportion of essential fatty acids than land plants. Seaweed has high fiber content, making up 32% to 50% of dry matter. The soluble fiber fraction accounts for 51-56% of total fibers in green (ulvans) and red algae (agars, carrageenans and xylans) and 67-87% in brown algae (laminaria, fucus, and others). Soluble fibers are generally associated with having cholesterol-lowering and hypoglycemic effects (Dharmananda, 2002).

There are many studies on gelation of fish protein such as research for tilapia snack production; study of factors influencing the gel-forming process of fish protein; research to improve gel-forming ability; studies on surimi production; research into food production from fish protein gel (fish balls, surimi, fish cakes, ...). However, the studies on the production of combined fish protein and seaweeds are still limited. In this study, a new value-added product from pangasius by-product and seaweed was developed in snack form.

2. Materials and Methods

2.1. Materials

Pangasius by-products in the fillet process were purchased at GODACO (My Tho City, Tien Giang Province, Vietnam), frozen by ice and transported to Nong Lam university in 3 - 3.5 hours.

Seaweed is grinded and used in dried form (Ao Nori brand, Japan) (Figure 1).

Figure 1. The pangasius by-product after removing fat, skin and bones (A) and dried seaweed used for food (B).

2.2. Methods

2.2.1. Gel preparation

Pangasius by-product (after removing fat, skin and bones) was washed, drained and minced with meat grinder.

Fish by-product (1 kg) was minced for 30 seconds and kept cool during the grinding process by adding 10% - 15% of clean ice. Salt (NaCl) 0.1% was added to the by-product to increase the gelling effect of the product. Seaweed at 0, 1 and 3% was also added before grinding step. Spice was also added to the paste at 3 different ratio as following:

- 0.2% salt : 0.3% sugar : 0.1% MSG : 0.1% chili powder
- 0.5% salt: 0,5% sugar: 0.1% MSG: 0.1% chili powder
- 0.2% salt: 0.3% sugar: 0,1% MSG: 0.1% chili powder

Minced fish by-product (size 30 × 30 mm, the thickness of 2 mm and an average weight of 1.65

g) was cooked at 90°C for 5 min. After cooking, the fish cake was dried at 3 different conditions at 80°C for 105 min; 70°C for 150 min and 60°C for 200 min. Finally, dried fish cake was fried in shortening at 180°C for 10, 15, 20 and 30 sec (Figure 2).

Figure 2. Pangasius by-products was minced to create gel.

2.2.2. Texture profile analysis (TPA)

Fish gels were cut into cylinder (30 mm in diameter and 2 mm thick). Brookfield food texture analyzer (CT3, USA) was used to test for TPA. TPA was measured by a cylindrical-shaped probe, 12.7 mm in diameter. Fish samples were compressed at 1 mm/s to 75% of their original height.The sample was compressed to 75% of height.

2.2.3. Water hold capacity (WHC)

WHC of fish gels was determined by centrifugation (Z 216 MK, Germany). Sample was wrapped in filter paper to absorb water then centrifuged at speed of 6000 r/min in 20 min. Samples were weighed before and after centrifugation to calculate WHC as following:

$$\text{WHC}(\%) = \frac{W_0 – \Delta W}{W_0} \times 100 \text{ (Skipnes et al., 2007)}$$

With:

$$W_0 = \frac{v_0}{v_0 + D_0} \times 100$$

and

$$\Delta W = \frac{\Delta v_0}{v_0 + D_0} \times 100$$

v_0: Initial water content of the sample

Δv_0: Difference in water content of the sample, before and after centrifugation

D_0: Initial dry mass of the sample. The dry mass can be determined gravimetrically.

2.2.4. Moisture

About 100 g of sample was analysed for moisture by using the moisture analyzer (AND MX-50, Japan) after drying. Each treatment was analysed in triplication.

2.2.5. Sensory analysis

The sensory of final products was investigated following the method of Ha (2006). Sensory experiments were arranged in a well-ventilated room with no strange smell, sufficient light, sensory time from 8 - 11 h, the age of the panels from 18 to 23 years old, the ratio of male to female is 1:1. The test sample is placed on a white porcelain plate, each of which is encoded with 3 random digits. The evaluation score following 9-point category scale.

2.2.6. Statistical analysis

All analytical measurements were carried out in triplications and the results were reported as mean values ± standard deviations. Data were analysed by one way analysis of variance (ANOVA) and least significant difference (LSD) using SPSS software version 21.0 (IBM Corp., United States).

3. Results and Discussion

The results on the texture of the product are showed in Table 1. The difference in hardness, cohesiveness, springiness, gumminess, and chewiness among the treatments was insignificant ($P > 0.05$). On average, NT1 had the highest hardness of 2.92 (N), and NT3 had the lowest hardness of 2.59 (N). For cohesiveness, NT3 has the highest cohesiveness of 0.83 compared to the rest of the treatment, the added seaweed may increase the cohesiveness of the product, but this increase was negligible. The springiness of NT1 was lowest (1.19 mm) and the highest was NT2 (1.28 mm), the addition of seaweed increased the springiness. However, the springiness of the sample decreased at 3% of seaweed addition. For gummi-

Table 1. Results of texture profile analysis of fish gels

Treatment	NT1 0% seaweed	NT2 1% seaweed	NT3 3% seaweed
Hardness (N)	2.92 ± 0.27	2.70 ± 0.16	2.59 ± 0.18
Cohesiveness	0.82 ± 0.06	0.82 ± 0.01	0.83 ± 0.36
Springiness (mm)	1.19 ± 0.09	1.28 ± 0.04	1.25 ± 0.07
Gumminess (N)	2.36 ± 0.17	2.22 ± 0.14	2.15 ± 0.21
Chewiness (mJ)	2.83 ± 0.25	2.85 ± 0.25	2.70 ± 0.22

Different letters (a, b, c) in the same row indicate significant differences ($P \leq 0.05$).

Table 2. Results of texture profile analysis of fish gels

Treatment	NT1 0% seaweed	NT2 1% seaweed	NT3 3% seaweed
WHC (%)	45.11^{a} ± 1.09	44.76^{a} ± 0.27	29.28^{b} ± 0.72

Different letters (a, b, c) in the same column indicate significant differences ($P \leq 0.05$).

ness, NT1 exhibited maximum gumminess (2.36 N). The percentage of seaweed added is inversely proportional to the gumminess with the larger the percentage of seaweed addition, the lower the gumminess. With an exerted force of 2.85 (mJ), NT2 had the highest chewiness and the lowest was NT3: 2.7 (mJ). The chewiness was the characteristic quantity for the solidity of food. The chewiness of NT2 was also higher than that of NT1. The addition of seaweed makes the texture of the sample more solid. However, when seaweed was added too much, it will affect the bonding of the protein gel. Therefore, the structure of gel was weakened and unstable leading to the lower chewiness of NT3 as compared to NT2.

In general, the addition of seaweed could change the texture of the samples, although there was no significant difference.

3.1. WHC of seaweed snack product

The results showed that the WHC value of NT1 (0% seaweed) and NT2 (1% seaweed) were similar (Table 2). However, the WHC of NT3 (3% seaweed) was significantly lower than NT1 and NT2 ($P < 0.05$).

WHC of fish gels was decreased with the increase in seaweed addition to the product. The decrease in WHC between treatments can be explained by seaweed addition prevented the formation of protein networks, making protein bondings unstable. The reduction of space gaps decreased the ability to hold water inside. It can be concluded that with a less stable spatial structure, NT3 had poor texture and not as stable as NT1 and NT2. From the results of TPA and WHC, NT2 (1% seaweed addition) was selected for the next step of our study.

3.2. Effects of seasoning ratio on sensory quality of snack product

The difference in sensory scores between the treatments was significant among treatments ($P < 0.05$). NT3 was highest with an average score of 6.95; NT2 had the lowest mean score of 5.65 (Table 3).

In this experiment, NT3 had the lowest salt addition of 0.2% compared to other treatments. However, the sensory quality of NT3 was most favoured by the panels. This was also under the tendency of consumers to reduce salt in processed products and in the diet, which was also recommended by WHO. Therefore, treatment 3 (0.2% salt: 0.3% sugar: 0.1% MSG: 0.1% chili powder) was used for seasoning of the product.

3.3. Effect of drying on the moisture of the product

As shown in Table 4, NT2 has the highest moisture of 8.49%. NT1 and NT2 had the same moisture of 8.17% and significantly lower than NT2 ($P < 0.05$). In the drying process, the hot air reduces the relative moisture of the medium, increasing driving force for the drying process. In this period, the heated material and the higher temperature of the drying medium increases the rate of water diffusion from the inside of the material to the surface of the drying material and into the environment, by molecular diffusion leading to the reduction the moisture of the product.

Table 3. Results on sensory test of snack product

Treatment	NT1 (0.3% salt: 0.5% sugar: 0.1% MSG: 0.1% chili powder)	NT2 (0.5% salt: 0.5% sugar: 0.1% MSG: 0.1% chili powder)	NT3 (0.2% salt: 0.3% sugar: 0.1% MSG: 0.1% chili powder)
Taste	$5.75^{b} \pm 0.71$	$5.65^{c} \pm 1.18$	$6.95^{a} \pm 0.60$

Different letters (a, b, c) in the same row indicate significant differences ($P \leq 0.05$).

Table 4. Moisture of product after drying

Treatment	NT1 (80°C, 105 min)	NT2 (70°C, 150 min)	NT3 (60°C, 200 min)
WHC (%)	$45.11^{a} \pm 1.09$	$44.76^{a} \pm 0.27$	$29.28^{b} \pm 0.72$

Different letters (a, b, c) in the same row indicate significant differences ($P \leq 0.05$).

Table 5. Sensory results of snack product at different frying conditions

Treatment	NT1 (10 sec)	NT2 (15 sec)	NT3 (20 sec)	NT4 (30 sec)
Smell	$5.25^{c} \pm 0.44$	$6.30^{b} \pm 0.47$	$7.40^{a} \pm 0.50$	$4.00^{d} \pm 0.00$
Taste	4.25c ± 0.44	6.25b ± 0.44	$7.30^{a} \pm 0.57$	$3.35^{d} \pm 0.48$
Colour	$5.10^{c} \pm 0.55$	$5.80^{b} \pm 0.61$	$7.30^{a} \pm 0.80$	$3.33^{d} \pm 0.57$
Texture	$4.25^{c} \pm 0.63$	$6.25^{b} \pm 0.44$	$7.45^{a} \pm 0.32$	3.20d ± 0.41
Overall score	$4.70^{c} \pm 0.47$	$6.20^{b} \pm 0.41$	$7.75^{c} \pm 0.44$	$3.50^{d} \pm 0.51$

Different letters (a, b, c) in the same row indicate significant differences ($P \leq 0.05$).

This moisture reduction also changed the texture of the treatments from the original texture of the gel form to a dry and hard texture (Figure 3). With significant shorter drying time as compared to NT3, drying condition at NT1: 80°C and 105 min was chosen for the product.

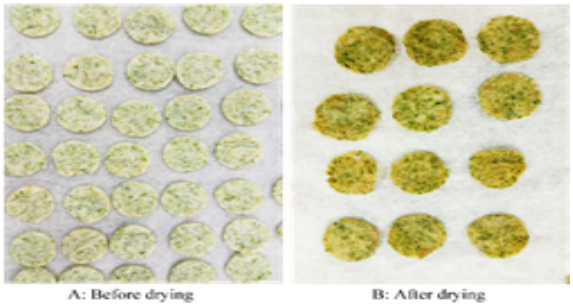

Figure 3. Samples before drying (A) and after drying (B), with changes in color, mass, moisture and texture.

3.4. Effect of frying condition on sensory quality of product

The results of the sensory evaluation are showed in Table 5. The difference between the treatments was significant ($P < 0.05$). The frying process changed the color, smell, taste, texture and moisture content of products. The 20-second frying treatment has the highest overall score (7.75) and the lowest overall score of 30 seconds (3.50). The sensory score of the frying treatment at 20 seconds in terms of smell, taste, texture and color were 7.40, 7.30, 7.45 and 7.30, respectively.

The temperature of 180°C (greater than 120°C) is the condition for phase 3 reaction of the Maillard reaction. High temperature accelerates the chemical reaction and increases the rate of water evaporation in the food thereby speeding up the maillard Maillard reaction. When frying for a long time, the amino acids and sugars are converted to aldehydes, nitrogen-containing compounds, and alcohols condensate, making food bitter and dark (Mallawaarachchi., 2017) (Figure 4). These conditions also changesd the sensory quality of the samples.

The treatment with the shortest time of 10 sec (NT1) had a brighter color and typical flavor of the fried product, but had the toughest texture. Because the frying time was not enough to completely removed the water, so the texture of the product was not crispy and toughed.

For NT3 frying at 20 sec, the score results

Figure 4. Product color at 180°C and different frying time (from left to right): 10, 15, 20 and 30 sec.

showed a superior in smell, color, taste and texture as compared to other treatments (Figure 5). Thus, frying time at 20 sec is selected. This frying time is enough to ensure the good quality of the product. If frying time is shorter than 20 sec, the water is not completely removed from the sample and reduced the crispy of product. If frying time is longer than 20 sec, the color is dark and bitter with a burnt odor which is not suitable for consumption.

Figure 5. Final seaweed pangasius snack product.

4. Conclusions

The results showed that seaweed-added products resulted in better texture as compared to seaweed non-added products. However, seaweed addition > 3% weakened the product's texture. The appropriate seaweed ratio was 1%. The appropriate spice ratio was 0.2% salt: 0,3% sugar: 0.1% MSG: 0.1% chili powder: 0.1% seaweed flavor powder and vegetable oil. The product (size 30 × 30 mm and thickness of 2 mm) was dried at 80°C in 105 min to the final moisture of 8,17%. The product was fried by shortening for 20 sec at 180°C resulted in the highest sensory value. For further study, more combination of spices should be investigated to create fish snack product with diverse taste to meet the demands of customer

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Fish composition in Dong Nai biosphere reserve in Vietnam

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ABSTRACT

Dong Nai biosphere reserve (DNBR) is well known for its high level of biodiversity and of global meaningful ecosystem. The fauna includes 84 species of mammals belonging to 28 families, 10 orders; 407 bird species; 141 reptile and amphibian species; 175 fish species; 2,017 insect species. The fish fauna of DNBR maintains many rare and endangered fish species recorded in the Vietnam red book and international union for conservation of nature red list (IUCN's red list) such as *Scleropages formosus* and many other rare fish species, such as *Morulius chrysophekadion*, *Chitala ornata*, *Probarbus jullieni*, *Cyclocheilichthys enoplos*... This study was aimed to identify fish composition distributed in DNBR. After the sampling period (01/2019 to 08/2019), a total of 114 fish species belonging to 11 orders and 28 families were recorded in DNBR. There were 09 species of fish on the list of rare and endangered fish species of Ministry of Agriculture and Rural Development of Vietnam, 3 species (*Chitala ornata*, *Cosmochilus harmandi* and *Hemibagus filamentus*) on the Vietnam red list book; 01 species (*Ompok bimaculatus*) on the IUCN's red list, 11 exotic species, 78 commercial species and 13 species having potential as aquarium fish. In addition, the study also found the first presence of a species of phallostethid, Phenacostethus smithi in DNBR.

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1. Introduction

Dong Nai biosphere reserve (DNBR) is well known for its high level of biodiversity and of global meaningful ecosystem. The flora of DNBR includes 2,812 species of vascular plants belonging to 192 families, 99 orders. While the fauna includes 110 species of mammals belonging to 31 families, 12 orders; 348 bird species; 134 reptile and amphibian species; 175 fish species; 1,243 insect species (DNDNRE, 2017). The fish fauna of DNBR maintains many rare and endangered fish species recorded in the Vietnam red book and IUCN's red list such as *Scleropages formosus*, and high economic fish species included *Anguilla marmorata*, *Hemibagrus wyckioides*, *Oxyeleotris marmoratus*, and many other rare fish species, such as *Morulius chrysophekadion*, *Chitala ornata*, *Probarbus jullieni*, *Cyclocheilichthys enoplos*... (Nguyen et al., 2009). However, this aquatic resource is facing many threats and is in danger of extinction if there are no timely and effective conservation and management measures. Main threats includes overexploitation, destructive exploitation such as using of electric fishing gears, small mesh nets, explosives... destruction of spawning and nursing grounds. In addition, the presence of a large number of exotic species, such as Cichla ocellaris, Pterygoplichthys disjunctivus, Colosoma brachypomus ... also greatly affects the natural aquatic resources in DNBR through competition for food and habitats, and predators of native species... In addition, DNBR also has many species of fish with colorful, beautiful shape

and unique which have great potential as aquarium fish. Therefore, the study on fish species with potential as ornamental fish helps to re-evaluate the value of these species and propose an efficient exploitation and use of biological resources contributing to increase income as well as to diverse livelihoods for people. Therefore, an up-to-date study of fish species composition at DNBR is necessary to provide an important scientific basis for the identification of rare species, endangered species, economic fish species, fish species are valuable for ornamental purposes. On that basis, proposing measures to conserve endangered species, protect and develop economic fish species, and efficiently use fish species of ornamental value. From there, helping people living in DNBR to develop economic, improve their livelihoods, as well as raise awareness in the sustainable use of aquatic resources and conservation of rare species.

2. Materials and Methods

The study on diversity of fish at DNBR (Figure 1) was conducted from January 2019 to August 2019.

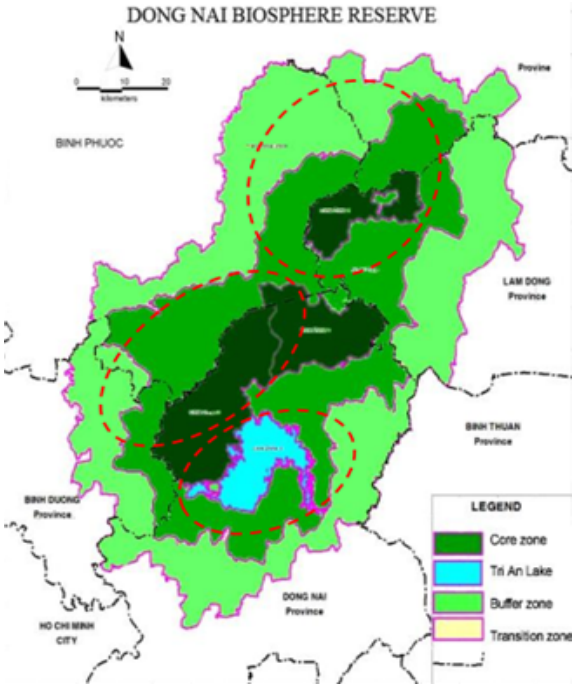

Figure 1. Sampling areas at the Dong Nai biosphere reserve.

The secondary data were collected at DNBR, Dong Nai Aquatic Resource Protection Department, Dong Nai Department of Agriculture and Rural Development ... as the basis for field investigation and survey. The content of collected data included the number of fishermen and registered fishing gears, the main fishing grounds, fish species composition and the annual catches on Tri An reservoir.

Primary data were collected through daily fishing logs of 30 fishermen (for 9 months). In addition, these fishermen were instructed on the sampling procedure (fish species that does not exist in the color photo album of fish species identified at DNBR) and how to fix fish samples in plastic bottles (5 L) containing formalin (10%). The content of collected data included species composition and catch yield of each species. The yield and species composition data were also collected from the fishing wharfs through interviews and copy the buying and selling notebooks of the owner, in addition to sorting, photographing and sampling the fish species there.

In addition, the sampling was conducted in different types of water bodies of the typical aquatic ecosystems of DNBR included Suoi Rang, Suoi Samach, Suoi Da Dung, Suoi Cop, Ba Hau, Tri An reservoir and Ramsar Bau Sau (Nam Cat Tien National Park) using dip nets, fishing net, fishing trap...

Fish samples were weighed and measured (according to the instructions of Pravdin, 1973). Fish samples were then photographed in the field, labeled with a local name, time, location, inserted into the mouth or gill, and stored in 10% formalin solution. Fish specimens were transferred to the laboratory of Faculty of Fisheries, Nong Lam University for analysis.

Fish were identified by measuring and counting different morphological parameters including total length, standard length, dorsal fin, pelvic fin, pectoral fin, lateral scale number... based on the taxonomic keys published by Vidthayanon (2008), Vasil'eva et al. (2013) and Nelson et al. (2016).

3. Results and Discussion

A total of 114 fish species belonging to 11 orders and 28 families were recorded (Table 1). These species belong to 28 families and 11 orders in which the Cypriniformes was the most abundant order with 50 species accounting for 43.9%

followed by the Perciformes and Siluriformes with 24 species (20%) and 21 species (18.4%), respectively (Figure 2).

Table 1. The fish species composition in DNBR, Dong Nai (2019)

	Scientific name
	Osteoglossiformes
	Notopteridae
1	*Chitala ornata* (Gray, 1831)
2	*Notopterus notopterus* (Pallas, 1780)
	Clupeiformes
	Clupeidae
3	*Corica laciniata* Fowler, 1935
4	*Clupeichthys aesarnensis* Wongratana, 1983
5	*Clupeoides borneensis* Bleeker, 1852
	Cypriniformes
	Balitoridae
6	*Nemacheilus platiceps* Kottelat, 1990
	Cobitidae
7	*Acantopsis dialuzoha* van Hasselt, 1823
8	*Lepidocephalichthys hasselti* (C&V, 1846)
	Cyprinidae
9	*Barbonymus gonionotus* Bleeker, 1849
10	*Barbonymus schwanenfeldi* Bleeker, 1854
11	*Barbonymus altus* Gunther, 1868
12	*Cosmochilus harmandi* Sauvage, 1878
13	*Chela laubuca* (Hamilton, 1822)
14	*Cirrhinus microlepis* Sauvage, 1878
15	*Crossocheilus reticulatus* Fowler, 1934
16	*Danio pulcher* Blyth, 1860
17	*Ctenopharyngodon idella* Valenciennes, 1844
18	*Cyclocheilichthys armatus* Valenciennes, 1842
19	*Cyclocheilichthys enoplos* Bleeker, 1849
20	*Cyclocheilichthys repasson* Bleeker, 1853
21	*Cyclocheilichthys apogon* Valenciennes, 1842
22	*Cyclocheilichthys* cf. *lagleri* Sontirat, 1989
23	*Cyprinus carpio* Linnaeus, 1758
24	*Leptobarbus hoevenii* Smith,1945
25	*Poropuntius deauratus* (Valenciennes, 1842)
26	*Puntius* cf. *brevis* Bleeker, 1806
27	*Puntius rhombeus* Kottelat, 2000
28	*Hampala macrolepidota* van Hasselt, 1823
29	*Henicorhynchus caudimaculatus* Fowler, 1934
30	*Henicorhynchus lobatus* Smith, 1945
31	*Henicorhynchus siamensis* Sauvage, 1881
32	*Labiobarbus lineatus* Smith,1945
33	*Labiobarbus siamensis* Sauvage, 1881
34	*Thynnichthys thynnoides* Bleeker, 1852
35	*Hypophthalmichthys molitrix* Valenciennes, 1844
36	*Hypophthalmichthys nobilis* Richardson, 1845
37	*Labeo chrysophekadion* Bleeker, 1849
38	*Labeo rohita* Hamilton, 1822
39	*Osteochilus hasselti* Valenciennes, 1842
40	*Osteochilus lini* Fowler, 1935
41	*Osteochilus waandersi* (Bleeker, 1852)
42	*Osteochilus microcephalus* Valenciennes, 1842
43	*Paralaubuca barroni* Fowler, 1934
44	*Parachela maculicauda* Smith, 1934
45	*Puntioplites falcifer* Smith, 1929
46	*Puntioplites proctozysron* Bleeker, 1865
47	*Puntius orphoides* Valenciennes, 1842
48	*Rasbora trilineata* Steindachner, 1870
49	*Esomus metallicus* Ahl, 1923
50	*Rasbora paviana syn. R. paviei* Tirant, 1885
51	*Rasbora borapetensis* Smith, 1934
52	*Scaphognathops stejnegeri* Smith, 1931
53	*Mystacoleucus marginatus* Valenciennes, 1842
54	*Systomus aurotaeniatus* Tirant, 1885
	Gyrinocheilidae
55	*Gyrinocheilus aymonieri* Tirant, 1883
	Siluriformes
	Bagridae
56	*Hemibagrus nemurus* (Valenciennes, 1839)
57	*Hemibagrus wyckioides* (Fang & Chaux, 1949)
58	*Hemibagrus filamentus* (Fang & Chaux, 1949)
59	*Mystus albolineatus* Roberts, 1994
60	*Mystus rhegma* Fowler, 1935
61	*Mystus mysticetus* Roberts, 1992
62	*Mystus nemurus* Valenciennes, 1840
63	*Mystus singaringan* Bleeker, 1846
64	*Pseudomystus siamensis* Regan, 1913
	Bagriichthidae
65	*Bagrichthys obscurus* Ng, 1999
	Clariidae
66	*Clarias batrachus* Linnaeus, 1758
67	*Clarias gariepinus* Burchell, 1822

Table 1. The fish species composition in DNBR, Dong Nai (2019, con't)

	Scientific name
68	*Clarias macrocephalus* Gunther, 1864
	Loricariidae
69	*Pterygoplichthys disjunctivus* Weber, 1991
	Pangasiidae
70	*Pangasius macronema* Bleeker, 1850
	Akysidae
71	*Akysis maculipinnis* Fowler, 1934
	Siluridae
72	*Micronema bleekeri* Bocourt, 1866
73	*Micronema apogon* Bleeker, 1851
74	*Ompok siluroides* Lacepede, 1803
75	*Kryptopterus* sp.
76	*Wallago attu* Bloch & Schneider, 1801
	Beloniformes
	Belonidae
77	*Xenentodon cancila* Hamilton, 1822
78	*Xenentodon canciloides* Bleeker, 1854
	Hemiramphidae
79	*Dermogenys siamensis* Fowler, 1934
80	*Hyporhamphus limbatus* Valenciennes, 1847
81	*Zenarchopterus ectuntio* Hamilton, 1822
82	*Dermogenys pusilla* Kuhl & van Hasselt, 1823
	Synbranchiformes
	Mastacembelidae
83	*Mastacembelus* cf. *circumcinctus* Hora, 1924
84	*Mastacembelus armatus* (Lacepede,1800)
85	*Macrognathus siamensis* Gunther, 1861
86	*Mastacembelus favus* Hora, 1923
	Synbranchiformes
	Synbranchidae
87	*Monopterus albus* Zuiew, 1793
	Perciformes
	Anabantidae
88	*Anabas testudineus* Bloch, 1792
	Ambassidae
89	*Parambassis siamensis* Fowler, 1937
90	*Parambassis apogonoides* Bleeker, 1851
91	*Parambassis wolffi* Bleeker, 1850
	Channidae
92	*Channa lucius* Cuvier, 1831
93	*Channa striata* Bloch, 1793
94	*Channa* cf. *gachua* Hamilton, 1822
	Cichlidae 95 *Cichla ocellaris* Bloch & Schneider, 1801
96	*Oreochromis mossambicus* Peters, 1852

Table 1. The fish species composition in DNBR, Dong Nai (2019, con't)

	Scientific name
97	*Oreochromis niloticus* Linnaeus, 1758
98	*Oreochromis* red hybrid
	Eleotridae
99	*Oxyeleotris marmorata* Bleeker, 1852
	Gobiidae
100	Brachygobius sabanus Inger, 1958
101	Glossogobius aureus Akihito & Meguro, 1975
102	Glossogobius giuris (Hamilton,1822)
103	Papuligobius ocellatus (Fowler, 1937)
104	Gobiopterus cf. chuno Hamilton, 1822
105	Brachygobius cf. nunus Hamilton, 1822
	Helostomatidae
106	*Helostoma temminckii* Cuvier, 1829
	Pristolepididae
107	*Pristolepis fasciata* Bleeker, 1851
	Belontiidae
108	*Trichopodus microlepis* Gunther, 1861
109	*Trichopsis vittata* Cuvier, 1831
110	*Trichopodus trichopterus* Pallas, 1770
111	*Betta prima* Kottelat, 1994
	Tetrodontiformes
	Tetraodontidae
112	*Monotrete leiurus* (Bleeker, 1850) syn. *Tetraodon leiurus* (Bleeker,1951)
113	*Carinotetraodon lorteri* (Tirant, 1885)
	Atheriniformes
	Phallostethidae
114	*Phallostethus* cf. *smithi*

The total number of species found in this study was more than that recorded by Nguyen (1987), Nguyen et al. (2005) and Nguyen et al. (2009) who reported the total number of 102, 109, 67 and 99 species, respectively (Table 2). The reason for the high number of species found in this study could be due to the combination of different research methods: photo-list interviews, indirect sampling from fishing wharfs and fishermen, and direct sampling for a long time on the field from different types of water bodies, especially from the restricted and prohibited fishing areas for commercial purposes.

The results noted the presence of two freshwater pufferfish species *Monotrete leiurus* (Bleeker, 1850) and *Carinotetraodon lorteri* (Tirant, 1885) which were not recorded by Nguyen et al. (2009). In addition, during field survey, a species of phallostethid fishes, *Phenacostethus smithi*, was col-

lected in the slow-flowing water body near to Tri An reservoir. This finding is very important and significant because this is the first time this species has been recorded in DNBR. In addition, the phallostethid fauna has not been well studied in Vietnam, especially in Dong Nai province. On the other hand, *Scleropages formosus*, *Wallago micropogon* and *Anguilla marmorata* are rare and high economic value species of DNBR. However, of all the field sampling trips in this study, none of individual of these species were collected.

Table 2. Similarity and difference in species number and composition between this study and previous studies

Author	No of species
Nguyen (1987)	102
Nguyen et al. (2005)	109
Nguyen (2005)	67
Nguyen et al. (2009)	99
Present study	114

Out of 114 recorded species, there were 09 species of fish on the list of rare and endangered fish species of Ministry of Agriculture and Rural Development of Vietnam (MARD, 2008), 3 species (*Chitala ornata*, *Cosmochilus harmandi* and *Hemibagus filamentus*) on the Vietnam red list book (MOST, 2007), 01 species (*Ompok bimaculatus*) on the IUCN Red List (2015). *Ompok bimaculatus* (CR), *Clarias batrachus* (CR) *Chitala ornata* (EN) are in danger of extinction. The remaining species are in threaten of extinction if there are no timely and effective conservation and management measures (Table 3). Survey data also showed that *Ompok bimaculatus*, *Clarias batrachus*, *Cosmochilus harmandi*, *Hampala macrolepidota*, *Morulius chrysophekadion*, *Hemibagrus filamentus*, *Hemibagrus wyckioides* and *Gyrinocheilus aymonieri* were in high demand despite their high selling price. The main reasons are that these fish have delicious meat and very few tiny bones. The supply of these fish to the market mainly comes from wild fishing. However, the results of this study confirmed the finding of previous studies that the yield of these species is decreasing rapidly and the size of catches fish is very small as compared to before. The main causes of this decrease are overfishing, using destructive fishing gears, such as electric pulses, small mesh nets, explosives... destroying spawning and nursing grounds. Therefore, it is necessary to apply strict regulations and sanctions to protect and conserve these species, such as banning destructive fishing gears, regulating the age (or size) of fish for catching, regulating the mesh size, regulating the fishing season, restricting or prohibiting seasonal fishing at spawning and nursing grounds... In addition, the diversification of cultured species also contributes to reducing the pressure of wild fishing. Therefore, it is necessary to develop a small-scale aquaculture system to culture these species to not only reduce the pressure of exploiting them in the wild, but also create more jobs and improve the income of the people living in DNBR. However, culture of these species for commercial purpose should rely on artificial seed and artificial feed, avoid using trash fish as feed. In addition, the annual release of artificial fingerlings also helps to restore their populations in the wild. Further, it is essential to establish conservation zones for indigenous aquatic species, endangered species in each ecological zone.

Contrary to the state of these species, the survey results showed that the catch of *Chitala ornata* at fish wharfs was relatively high with sizes of catches fish quite big. However, these individuals may not be pure and endemic species of DNBR but possibly crossbred species escaping from fish cages. The crossbred species has no conservation value. Therefore, it is necessary to examine the genes of the captured fish to determine whether it is endemic or not as well as their distribution area for conservation purposes.

Survey results also recorded the appearance of 11 exotic fish species (Table 4). The results at fishing wharfs indicated that the catches of these species were quite high, especially *Cyprinus carpio*, *Aristichthys nobilis*, *Hypostomus plecoftomus*, *Hypophthalmichthys molitrix*, *Oreochromis niloticus*... These species are imported into Vietnam for different purposes: to diversify cultured species such as *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Aristichthys nobilis*, *Hypophthalmichthys molitrix*, *Labeo rohita*..., and to serve as ornamental species (*Cichla ocellaris*, *Hypostomus plecoftomus*...). Most alien species are omnivores fish which are well adapted and easily reproduce naturally in new habitats. Therefore, populations of these species grow very rapidly in the natural waters of DNBR. The increase in populations of *Cyprinus carpio*, *Ctenopharyngodon idellus*, *Aristichthys nobilis*, *Hypophthalmichthys molitrix*, *Labeo rohita*,

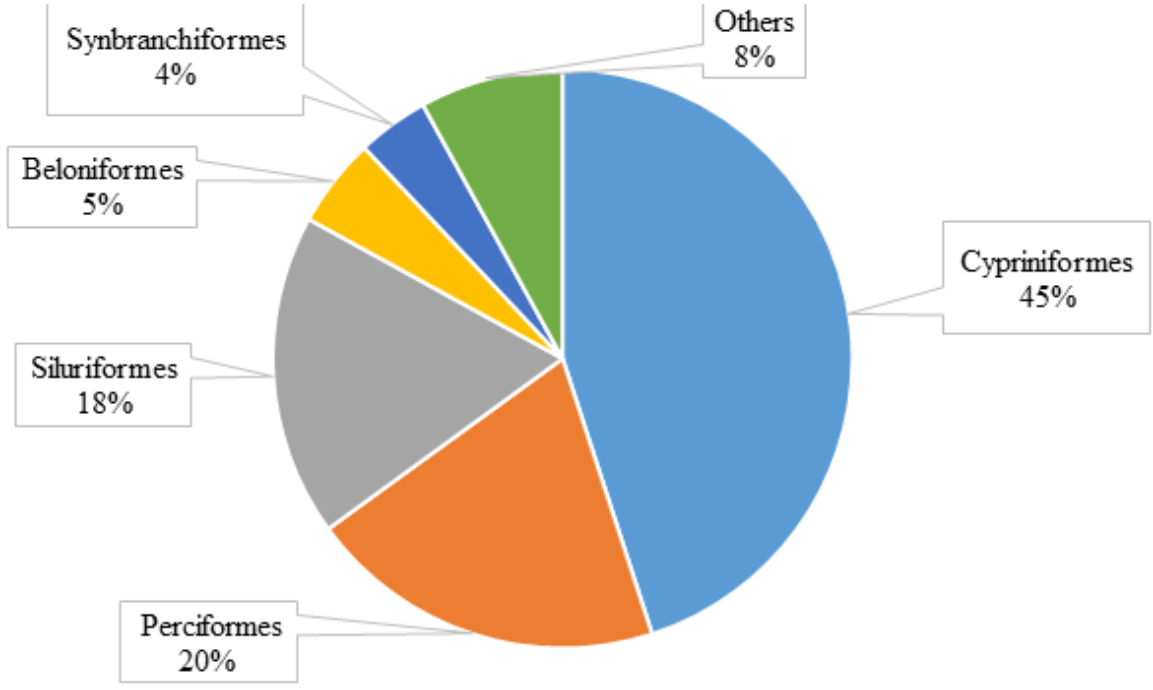

Figure 2. Fish species composition recorded at Dong Nai biosphere reserve.

Table 3. List of rare and endangered fish species[1]

	Scientific name	MARD (QD-82/2008) (*)	VRB-2007 (*)	IUCN-2020 (*)
1	*Ompok bimaculatus* Bloch, 1794	CR		NT
2	*Clarias batrachus* Linnaeus, 1758	CR		LC
3	Chitala ornata Gray, 1831	EN	VU	LC
4	Cosmochilus harmandi Sauvage,1878	VU	VU	LC
5	Hampala macrolepidota Kuhl&Van, 1823	VU		LC
6	Morulius chrysophekadion Bleeker,1850	VU		LC
7	Hemibagrus filamentus Fang&Chaux,1949	VU	VU	DD
8	Hemibagrus wyckioides Chaux&Fang, 1949	NT		LC
9	Gyrinocheilus aymonieri Tirant, 1883	VU		LC

[1]Sources: MARD (2008), Vietnam red list book (MOST, 2007), IUCN's red list (2015).
*MARD: Ministry of Agriculture and Rural Development; VRB: Vietnam red list book; IUCN: International union for conservation of nature; CR: critically endangered; EN: endangered; VU: vulnerable; NT: near threatened (includes LR/nt-lower risk/near threatened); LC: Lowest concern; DD: data deficient.

Helostoma temminckii... has contributed to increasing aquatic resources in DNBR, especially in Tri An reservoir, thereby improving income and stabilizing people's livelihoods. However, the overgrowth of species, such as *Hypostomus plecoftomus*, *Oreochromis niloticus*, *Oreochromis spp*... is considered a high risk to the populations of native fish species in term of food and habitat competition.

Survey results also recorded 78 economic fish species, accounting for 67.83% of the total fish species in DNBR. There were 10 species with an annual catches more than 200 tons/year including freshwater anchovy group (approx. 2500 tons/year, accounting for 35.3% of the total catch, mainly species of *Corica laciniata*, *Clupeichthys aesarnensis*, *Clupeoides borneensis*); Wrestling halfbeak group (1225 tons/year, accounting for 15.2% of the total catch, mainly

Table 4. List of exotic fish species

	Scientific name
1	*Cyprinus carpio* (Linnaeus, 1758)
2	*Ctenopharyngodon idellus* (Cuvier & Valenciennes, 1844)
3	*Aristichthys nobilis* (Richardson, 1844)
4	*Hypophthalmichthys molitrix* (Cuvier & Valenciennes, 1844)
5	*Labeo rohita* (Hamilton, 1822)
6	*Hypostomus plecoftomus* (Linnaeus, 1758)
7	*Oreochromis niloticus* (Linnaeus, 1757)
8	*Oreochromis* spp.
9	*Cichla ocellaris* Schneider, 1801
10	*Clarias gariepinus* (Burchell, 1822)
11	*Helostoma temminckii* (Cuvier, 1829)

Table 5. List of species with potential as aquarium fish

	Scientific name
1	*Channa* cf. *gachua* Hamilton, 1822
2	*Rasbora borapetensis* Smith, 1934
3	*Danio albolineatus* Blyth, 1860
4	*Trichopsis vittata* (Cuvier, 1831)
5	*Esomus metallicus* Ahl, 1924
6	*Rasbora trilineata* Steindachner, 1870
7	*Rasbora paviana* syn. *R. paviei* Tirant, 1885
8	*Chela laubuca* Hamilton, 1822
9	*Trichopodus trichopterus* Pallas, 1770
10	*Brachygobius nunus* Hamilton, 1822
11	*Hyporhamphus limbatus* Valenciennes, 1847
12	*Betta splendens* Regan, 1910
13	*Betta prima* Kottelat, 1994

species of *Dermogenys siamensis*, *Hyporhamphus limbatus*, *Zenarchopterus ectuntio*, *Dermogenys pusilla*); *Oreochromis mossambicus* (565 tons/year); *Cyprinus carpio* (412 tons/year); *Glossogobius giuris* (229 tons/year), etc. The results indicated that these species are the main fishing species and are contributing greatly to people's livelihoods. The results also showed that the catches of trash fish were relatively high at 574 tons/year. Trash fish is a by-product of fishing for higher value fish. Trash fish comprised the greatest amount of fish (Small mixed fish, low value fish, spoiled high value fish...) but included small molluscs, crustaceans... There are three terms for trash fish in Vietnamese: trash fish, trawler fish and pig fish, the latter being the lowest quality only and therefore having a more restricted meaning than the other two terms. The composition of trash fish will also vary depending on the type of gear set to fish but most is from trawling. Trawling fish is often used for direct feeding to farmed fish or livestock. The results also showed that there is conflicting uses for trash fish for different purposes such as: aquaculture feed, livestock feed and direct human food. Some species identified as trash fish are currently being used as human food with high consumption demand at the high prices, especially *Dermogenys siamensis*, *Hyporhamphus limbatus*, *Zenarchopterus ectuntio*, *Dermogenys pusilla* species. In recent years, the catch composition is changing dramatically, with the rise of trash fish, especially from trawling. Although fishing technology has improved with better targeting of high value species, trash fish biomass continues to increase. If this continues, it will deplete aquatic resources in the area and directly affect people's livelihoods.

On the other hand, the results noted that many species of fish in DNBR have been exploited

for the ornamental fish trade such as *Chitala ornata*, *Mastacembelus armatus*, *Mastacembelus favus*, *Pseudomystus siamensis*, *Gyrinocheilus pennocki*... There are also still many species that have great ornamental fish potential included *Channa* cf. *gachua*, *Rasbora borapetensis*, *Danio albolineatus*, *Brachygobius nunus*, *Hyporhamphus limbatus*, *Betta splendens*, *Betta prima*, *Nemacheilus* spp., *Lepidocephalichthys* sp., *Pangio* spp., *Barilius* cf. *koratensis*, (Table 5)... In addition, many of them have also been successfully bred in captivity and conserved for example *Channa* cf. *gachua*, *Chitala ornata*, *Mastacembelus armatus*, *Mastacembelus favus*, *Betta splendens*, *Betta prima*...

4. Conclusions

The total number of fish species recorded in Dong Nai biosphere reserve was 114 species belonging to 28 families and 11 orders. There were 09 species of fish on the list of rare and endangered fish species of Ministry of Agriculture and Rural Development of Vietnam, 3 species (*Chitala ornata*, *Cosmochilus harmandi* and *Hemibagus filamentus*) on the Vietnam red list book, 01 species (*Ompok bimaculatus*) on the international union for conservation of nature red list, 11 exotic species, 78 commercial species and 13 species having potential as aquarium fish. The study also found the first presence of a species of phallostethid, Phenacostethus smithi in Dong Nai biosphere reserve.

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Optimization of total phenolic extraction of *Chromolaena odorata* leaf for antifungal activity against plant pathogens

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ABSTRACT

Phenolic compounds are secondary metabolites mainly responsible for different pharmacological activities of the plant extracts. In this study, microwave-assisted extraction (MAE) variables were optimized for the extraction of the phenolic compounds from *Chromolaena odorata* (*C. odorata*) by using a central composite design (CCD) of response surface methodology (RSM). The optimized conditions were at 62°C for 3 min with solvent to feed ratio of 14:1 (mL/g) and ethanol concentration of 50% (v/v). The highest yield of total phenolic compounds was 75.76 mgGAE/g. Ethanol extract clearly showed antifungal activity through antifungal index. The antifungal effect *Phytophthora capsici*, *Fusarium oxysporum* and *Colletotrichum siamese* were 79.07%, 78.66% and 78.42%, in 0.5% of ethanol extract, respectively.

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1. Introduction

Chromolaena odorata (*C. odorata*) belongs to the Asteraceae family, its chemical composition contains many flavonoid compounds including flavones, flavonols, flavanons and chalcones. Flavonoids are the decisive ingredients for the biological activities which were proved to have strong antioxidant activity, combined with anti-inflammatory effects have increased effectiveness in wounds. *C. odorata* leaves also contain a high amount of essential oils. The main ingredients of *C. odorata* essential oil include: α-pinene, β-pinene, D-limonene, β-ocimene, Caryophyllene, Pregeijerene, Germacrene-D, δ-cadinene, α-copaene, Caryophyllene oxide, δ-humulene... (Moses et al., 2010). In addition, *C. odorata* also contains long-chain or cyclic polyunsaturated fatty acids. Phenolic compounds were metabolic products of arachidonic acid that were considered defense weapons against plant pathogens. Adeyemo (2018) reported that ethanolic extract of *C. odorata* showed the highest antifungal effects against

Phytophthora megakarya, followed by cold-water extracts. Zones of clearance range were from 15.00 mm to 32.00 mm for ethanolic extraction while cold water extract was from 5.00 mm to 30.00 mm at concentration from 1% to 5%.

Microwave-assisted extraction has been employed to recover phenolic compounds from *C. odorata* leaves. The MAE is a novel, unconventional extraction method that is more efficient in recovering quality yields with reduced extraction time and less solvent (Moses et al., 2010). The MAE is affected by different factors including microwave power, temperature, irradiation time, solvent concentration, and solvent to feed ratio. To optimize these factors, response surface methodology (RSM) was used as a statistical method to generate the predictive mathematical model that takes into account possible interactions between the factors under consideration (Khuri & Mukhopadhyay, 2010). A central composite design (CCD) had been employed herein because it is one of the most efficient types of RSM and is useful in avoiding experimental processes under extreme conditions, which can result in unsatisfactory results.

Although *C. odorata* has been used as a medicine in Vietnam, there are not many studies on optimizing the process of obtaining phenolic-rich extract from *C. odorata*, especially biodynamic agriculture in general, biopesticides for plant pathogenic fungi in particular. Dwijendra (2014) mentioned that the use of chemical pesticides have negative impacts on animals, humans, even the whole ecosystem. Therefore, one of the proposed solutions is to use compounds from plants, especially phenolic. These compounds are not only effective in controlling diseases in plants, but also sustainable with the ecosystem. According to Kumlachew et al. (2014), using *Ruta chalepensis* extract to inhibit the growth of anthracnose *Colletotrichum gloeosporioides* on mango. The extract reduced the development of the disease to less than 36%, while for the control the level of damage was 93.4%. Therefore, this study investigated the optimization of phenolic compounds from *C. odorata* leaves and analyzed inhibition of three plant pathogenic fungi strains. MAE is used to extract phenolics compounds from *C. odorata*. Extraction conditions including temperatures, times, ethanol concentrations and solvent to feed ratio were optimized using RSM at CCD. Then, the antifungal activity of plant extract against *P. capsici*, *F. oxysporum* and *C. siamense* was examined.

2. Materials and Methods

2.1. Plant materials, chemicals, and reagents

Fresh *C. odorata* was collected at the Research Institute for Biotechnology and Environment (RIBE), Nong Lam University Ho Chi Minh City. The leaves were washed with tap water, dried at 40°C for 3 days, pulverized to powder form, and screened to a particle size of 0.1 mm. The powdered sample was stored in a dark container at (25°C ± 2). The moisture content of the plant sample before extraction was lower than 12% water per dry sample.

Fungal strains including *P. capsici*, *F. oxysporum*, *C. siamense* were provided by the Research Institute of Biotechnology and Environment, Nong Lam University, Ho Chi Minh City.

Ethanol, Folin-Ciocalteu reagent, gallic acid, and sodium carbonate were purchased from Sigma Aldrich.

2.2. Phytochemical screening

2.2.1. Alkaloids

The presence of alkaloids in the ethanolic extract was examined by Dragendroff's test. About 1 mL of extract was taken and added 3-5 drops of Dragendorff's. Dragendorff's reagent was prepared by 1 mL mixing solution-1 containing 0.85 g of bismuth (III) nitrate and 10 mL acid acetic in 40 mL of water, 1 mL solution-2 containing 8 g potassium iodide in 20 mL water to a solution of glacial acetic acid (4 mL) in deionization water (14 mL). If orange-red precipitate is formed after 3 min, indicating the presence of alkaloids in the crude extract.

2.2.2. Saponins

The presence of saponins in the ethanolic extract was examined by Froth test. About 0.5 mg of the extract was mixed with 5 mL of distilled water in a test tube and vigorously shaken for 2 min. Foam which persisted for 30 min and doesn't disappear upon warming indicates the presence of saponins in the extract.

2.2.3. Flavonoids

The presence of flavonoids in the ethanolic extract was examined by Shinoda Test. About 10 mg of extract was added to pinch of magnesium and 3 drops of concentrated hydrochloric acid was added. Formation of orange-pink colour indicates the presence of flavonoids.

2.2.4. Phenolic compounds

The presence of phenolic compounds in the ethanolic extract was examined by lead acetate test. About 10 mg of extract was taken and 0.5 mL of 1% lead acetate solution was added and the formation of precipitate indicates the presence of tannins and phenolic compounds.

2.2.5. Tannins

The presence of tannins the ethanolic extract was examined by ferric chloride test. About 0.5 mg of the extract, ferric chloride 5% a solution was added. Formation of dark blue precipitate indicates the presence of tannins.

2.3. MAE of *C. odorata* leaves

In all the above experiments, whether the dried *C. odorata* leaves have uniform size, were sifted through the ϕ1 mm sieve. The weight of material for several extracts was 1 g. The extraction was conducted in a microwave-assisted separator (QLABPro Closed Vessel Microwave Digestion) at different temperatures, times, ethanol concentration and solvent to feed ratio. The filtrate was obtained after centrifugation at 25°C, for 15 min (Hermle). Then used for determination of phenolic contents.

2.4. Determination total phenolic compounds

Total phenolic compounds (TPC) was determined by the method of Alara et al (2019a) with minor modifications. 30 µL of *C.odorata* extract was mixed with 100 µL of Folin-Ciocalteu reagent and 3000 µL of deionization water for 5 min, then 300 µL of 20% Na_2CO_3 was added to the mixture, filled up to 5 mL. The reaction mixture was kept in a dark room for 120 min and the absorbance was measured at 735 nm using a UV/Vis spectrophotometer (Agilent, California). Gallic acid was used as a standard in the determination of TPC from *C. odorata* leaves (gallic acid equivalents; GAE). The concentration of *C. odorata* leaf extract was calculated in relation to the gallic acid standard curve. Then, TPC was calculated by Eq. (1). The analysis was repeated three times and the results were expressed as mean ± standard deviation.

$$\text{TPC} = \frac{\text{A}}{\text{Weight of dried sample used (g)}} \quad (1)$$

with:

A: Concentration (mg/mL) × volum of solvent used (mL)

2.5. Experimental design and statistical analysis

Optimization of total phenolic content was carried out using a CCD of RSM. Experimental design includes five levels of 4 independents variables: irradiation time (2 – 4 min), extraction temperature (50 – 70°C), solvent to feed ratio (8:1 – 14:1 mL/g), ethanol concentration (40 – 60% v/v) was designed with Minitab 16.1 Statistical Software® for a total of 31 runs. In exploration studies, the marginal values of the extraction factors were determined as shown in Table 1.

A total of 16 experiments at 2 levels (upper and lower), 8 star experiments and 7 center experiments were carried out. Each experiment was conducted three times and average results were obtained. Experimental data were analyzed by MiniTab 16. The mathematical model of the influence of independent variables on the dependent variable has a quadratic polynomial function as follows:

$$Y_k = B_0 + \sum_{j=1}^{4} B_j X_j + \sum_{i,j=1}^{4} B_{ij} X_j + \sum_{j=1}^{4} B_{jj} X_j^2$$

Yk: Dependent variable (k = 1 – 4)

Xi,j: The encoding factor of the independent variable affects Yk

B_0: Regression coefficient of 0 degree

B_j: Regression coefficient of 1 degree affects the variable X_i to Y_k

B_{ij}: Coefficients of simultaneous influence of variable X_j to Y_k

B_{ij}: Regression coefficient of degree 2 describes the effect of variable X_j^2 on Y_k

Table 1. Experimental design-matrix encoding the independent variables

Variable name	Levels of evidence					
Uncoded variable	Code variable	-α	-1	0	+1	+α
A: Irradiation time [min]	U_1	1	2	3	4	5
B: Extraction temperature [°C]	U_2	40	50	60	70	80
C: Solvent:feed ratio [mL:g]	U_4	5	8	11	14	17
D: Ethanol concentration [%]	U_4	30	40	50	60	70

α = 2, U_{min}, U_{max} is the lower bound (-1) and upper bound (+1) of the independent variable, U0 = (U_{min} + U_{max})/2 is the mean value of the upper and lower bound.

2.6. Antifungal activity screening test

2.6.1. Media preparation

Potato glucose agar (PGA) medium containing 20 g D-Glucose, 20 g Agar, 200 g potato and 1000 mL distilled water were prepared. After sterilization and addition of streptomycin (0.1%), the medium was poured in a petri dish.

2.6.2. Fungal preparation

Three fungal strains including *P. capsisi*, *F. oxyporum* and *C. siamense* were inoculated on PGA medium with antibiotic supplementation at a temperature of 25°C ± 1, monitored for 5 - 7 days until fungal filaments overgrown agar plates.

2.6.3. Transplantation and Mycelial growth

Fungal plates were prepared using a disc of 9 mm diameter carrying mold spores on the surface of a petri dish containing highly enriched PGA medium of different concentration of *C. odorata* (0.1%, 0.2%, 0.3%, 0.4%, 0,5% v/v). The plates after inoculation were incubated at 25°C ± 1. Mycelium growth was observed and evaluated until the mycelium had grown to the plate of the non-enriched medium by measuring the mean of the two perpendicular diameters passing between the plates. Antifungal activity was evaluated by the formula (Chang et al., 2000):

$$I = \left(1 - \frac{d}{dc}\right) \times 100$$

where I: antifungal index, d: diameter of petri dish treated with the extracts, dc: diameter growth of the control.

2.6.4. Data analysis

Data was analysis of variance (ANOVA). These values are expressed as the mean ± standard deviation of a triplicate measurement.

3. Results and Discussion

3.1. Phytochemical of *C. odorata* leaves extract

The results obtained from phytochemical screening show that alkaloid, flavonoid, tannin, phenol and saponin were detected in all the extracts irrespective of the solvent. (Table 2). Generally, ethanol extraction has better efficiency than water extraction. Due to ethanol is considered a universal solvent, as its molecular structure allows for the dissolving of both polar and nonpolar compounds (Mark, 2018).

Table 2. Active ingredients tested in extracts of *C. odorata*

Active agents	Water extract	Ethanol extract
Alkaloids	+	++
Flavonoids	+	++
Phenolics	+	++
Tannins	+	++
Saponins	++	+

++ moderately detected; + Detected but weak.

3.2. Effect of extraction conditions on phenolic compounds

The MAE process parameters were optimized to maximize TPC from *C. odorata* leaves (Table 3). In addition, the impact of each MAE parameter and their interactions were examined. The effects of extraction factors on the TPC were presented in the following function:

Y_{TPC}= -490.265 + 43.762A + 7.284B + 20.046C + 5.099D + 0.117BC - 5.715A^2 - 0.069B^2 - 0.932C^2 - 0.049D^2

Table 4 shows the analysis of variance

Table 3. Experimental results of total phenolic compounds (TPC)

No.	Microwave-assisted extraction variables				TPC [mgGAE/g]
	A: Irradiation time [min]	B: Extraction temperature [°C]	C: Solvent:feed ratio [mL/g]	D: Ethanol concentration [%]	
1	3	40	8	40	23.97
2	5	40	8	40	36.27
3	3	60	8	40	11.16
4	5	60	8	40	25.2
5	3	40	14	40	56.79
6	5	40	14	40	62.82
7	3	60	14	40	62.35
8	5	60	14	40	70.74
9	3	40	8	60	24.72
10	5	40	8	60	38.57
11	3	60	8	60	13.08
12	5	60	8	60	25.17
13	3	40	14	60	57.97
14	5	40	14	60	63.11
15	3	60	14	60	62.13
16	5	60	14	60	71.10
17	2	50	11	50	39.12
18	6	50	11	50	59.18
19	4	30	11	50	47.09
20	4	70	11	50	42.05
21	4	50	5	50	00.87
22	4	50	17	50	78.82
23	4	50	11	30	51.44
24	4	50	11	70	53.47
25	4	50	11	50	72.09
26	4	50	11	50	71.99
27	4	50	11	50	70.24
28	4	50	11	50	72.24
29	4	50	11	50	71.60
30	4	50	11	50	70.72
31	4	50	11	50	71.61

(ANOVA) results and coefficients for TPC. The experimental data were fitted to a quadratic model. The P-value less than 0.05 indicated that the model was statistically significant. The irradiation time, extraction temperature, solvent to feed ratio and ethanol concentration all showed a significant effect ($P < 0.05$) on the yield of TPC. The P-value of the model of incompatibility test (lack to fit) is 0.087, greater than 0.05. Therefore, the regression model is compatible with the experiment.

The analytical results showed that four factors significantly affect the phenolic extraction process. This result was consistent with the general trend of the extraction of bioactive compounds from plant materials (Alara et al., 2019a). The results also showed that all four extraction factors including irradiation time, extraction temperature, solvent to feed ratio and ethanol concentration interacted with each other and affected the target function. Specifically, the effect of irradiation time, extraction temperature, ethanol concentration increased gradually to a critical value and if it continued to increase, it will decrease the overall value of the target function was decreased (Figure 1a, 1c, 1e). Higher quality phenolic compounds are mostly obtained from the plant matrix by using a binary solvent mixture (ethanol-water) because it depends on the polar-

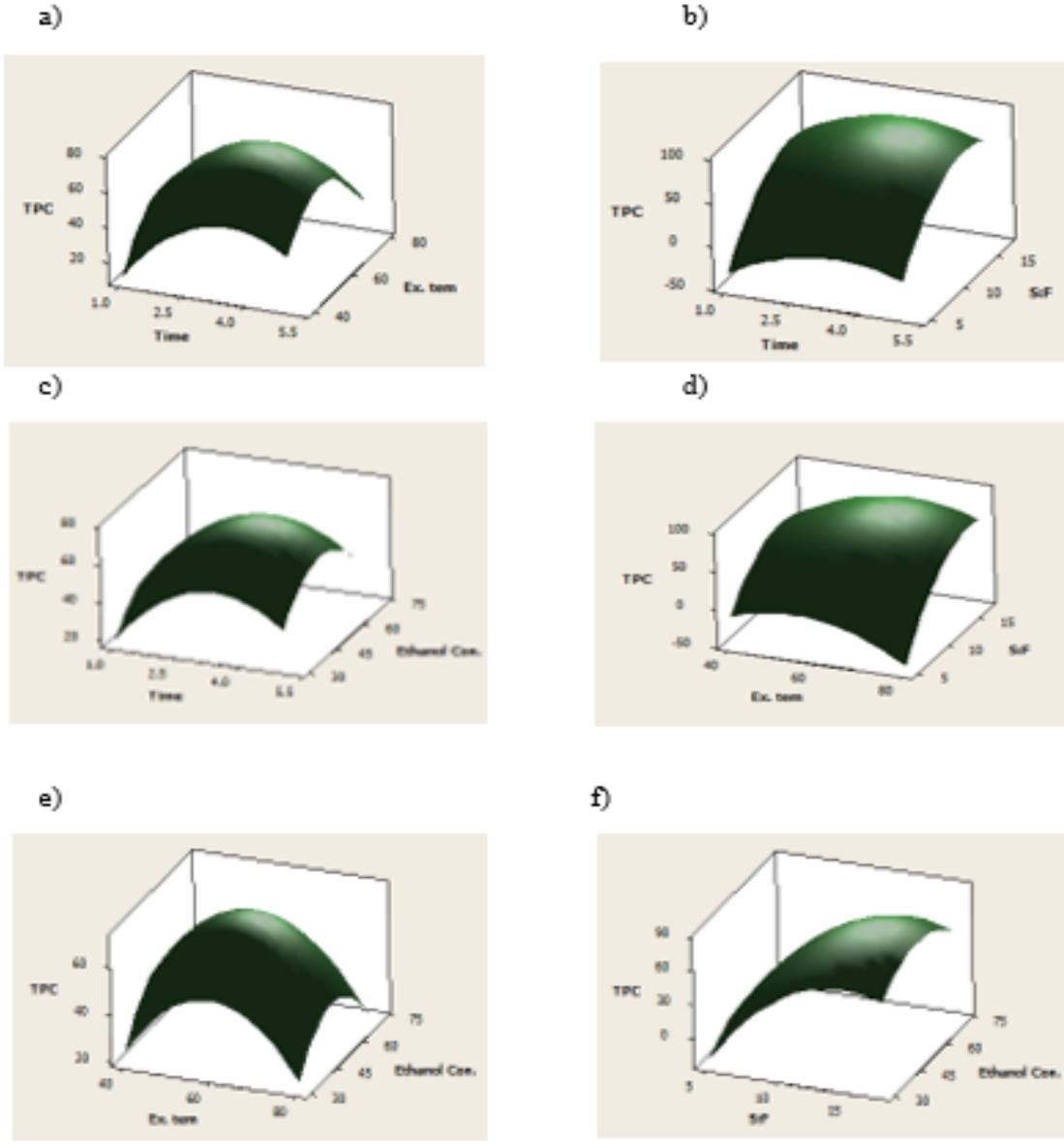

Figure 1. Response surface plots showing the influences of microwave-assisted extraction parameters on phenolic compounds. (a) irradiation time (A) versus extraction temperature (B) for total phenolic compounds (TPC); (b) A versus solvent:feed ratio (C) ; (c) A versus ethanol concentration (d) B versus C for TPC; (e) B versus D for TPC; (f) C versus D for TPC.

ity of solvents used (Alara et al., 2019a). Meanwhile, solvent to feed ratio has a positive effect on the target function (Figure 1b, 1d, 1f). Thus, from the results obtained it can be seen that within the research scope, when increasing irradiation time, extraction temperature, solvent:-feed ratio, ethanol concentration to an appropriate value will increased the value of the objective function. If an increase exceeded the optimal value, it decreased the value of the target function. For solvent feed ratio when reaching the optimum value, it did not increase the value of the target function if it continued to increase. The optimized conditions of TPC might be due to the polarity of the solvent used in extraction. A previous report showed that ethanol and water were environmentally friendly solvents that could extract a higher quantity of phenolic compounds

Table 4. Effects of the extraction factors on the target function by analysis of variance

Variable	df	TPC [mgGAEgDW^{-1}]	
		F value	P-value probability > F
Model	14	894.28	0.000
Linear			
Irradiation time (A)	1	302.01	0.000
Extraction temperature (B)	1	18.80	0.000
Solvent:feed ratio (C)	1	619.34	0.000
Ethanol concentration (D)	1	539.18	0.000
Interation			
AB	1	9.05	0.008
AC	1	1.56	0.230
AD	1	0.83	0.375
BC	1	183.76	0.000
BD	1	2.85	0.111
CD	1	0.08	0.785
Quadratic			
A^2	1	876.83	0.000
B^2	1	1263.56	0.000
C^2	1	1887.42	0.000
D^2	1	641.62	0.000
Lack to fit		3.14	0.087
R^2		99.87%	
Adjust R^2		99.76%	
Predicted R^2		99.35%	

from the plant matrix. In a similar study, Alara et al. (2019b) obtained a TPC of 88.52 mgGAE/g from *C. odorata* leaves under MAE optimized conditions of 51% (v/v) ethanol concentration, irradiation time of 3 min, temperature of 60°C and solvent-to-material ratio of 10:1 (mL/g).

In addition, the predictive models were verified by comparing the predicted and experimental data. The predicted conditions were highest at 62°C for 3 min with solvent to feed ratio of 14:1 (mL/g) and ethanol concentration of 50% (v/v). The results showed that the experimental values for TPC (75.76 mgGAE/g) did not vary significantly at the 5% level by using a paired t-test. Therefore, the regression equations derived from this study can be employed to optimize the extraction of phenolic compounds from *C. odorata* leaves.

3.3. Evaluate the antifungal ability of *C. odorata* extract

Total phenolic compounds of *C. odorata* leaves were extracted at optimum conditions then were evaporated to remove ethanol and prepared at 5 different concentrations, for evaluation of the antifungal activity against *P. capsici*, *F. oxysporum* and *C. siamense* (Table 5).

Antifungal effects increased with the increasing concentration of *C. odorata* extract from 0.1 to 0.5%. Maximum antifungal effects against *P. capsici* was found to be 79.65% in 0.5% ethanol extract clearly (Figure 2). For *F. oxysporum*, the ability to inhibit fungi at concentrations of 0.1%, 0.2% and 0.3% was not statistically significant between treatments, but at concentrations of 0.4% and 0.5% differed significantly between treatments (Figure 3). Similarly, *C. siamense*'s antifungal effect of the extracts was varying such as: (Table 4) 0; 41.67; 49.67; 56.67; 63.83; 65.76 mm at 0%, 0.1%, 0.2%, 0.3%, 0.4% and 0.5% respectively. Results showed that at all concentrations was a statistically significant difference (Figure 4). The interaction of polyphenols with nonspecific forces such as hydrogen bonding and hydrophobic effects, lipophilic forces, as well as by covalent bond formation, was related to microbial membranes, adhesions, enzymes, and cell envelope transport proteins (Cowan 1999; Kumar & Pandey 2013). The antibacterial activity of polyphenols may be

Table 5. Antifungal index (%) of *C. odorata extract*

Concentration (% v/v)	Antifungal index (%)		
	P. capsici (3 days)	*F. oxysporum* (5 days)	*C. siamense* (5 days)
NC	00.00^{e}	00.00^{d}	00.00^{f}
0.1%	61.63^{d}	58.23^{c}	53.33^{e}
0.2%	69.19^{c}	58.23^{c}	61.75^{d}
0.3%	75.58^{b}	58.54^{d}	69.12^{c}
0.4%	79.07^{a}	68.90^{b}	76.67^{b}
0.5%	79.65^{a}	78.66^{a}	78.42^{a}

In the same column, there is a statistically significant difference in values with different characters with $P < 0.05$. The percentage is converted by the formula $y = \sqrt{(x + 0.5)}$ before analysis ANOVA.

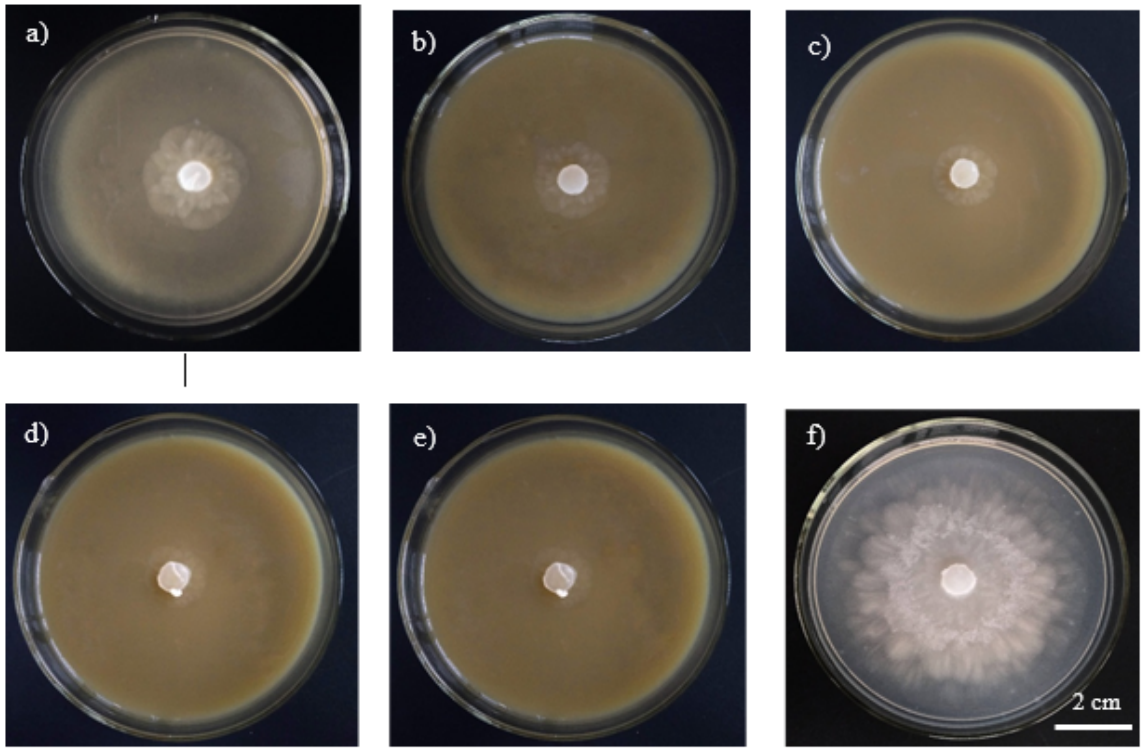

Figure 2. The antifungal ability of *C. odorata* extract against *P. capsici* at different concentration (a) 0.1% v/v; (b) 0.2% v/v; (c) 0.3%; (d) 0.4% v/v; (e) 0.5% v/v; (f) Negative Control (NC).

also due to the capacity of these compounds to chelate iron, vital for the survival of almost all bacteria (Field & Lettinga, 1992). Borges et al. (2013) reported that gallic and ferulic acids induce irreversible changes in *E. coli*, *P. aeruginosa*, *S. aureus*, and *L. monocytogenes* membrane properties. Ting (2013) investigated the antibacterial activity and membrane interaction of 5 flavonoids (kaempferol, quercetin, chrysin, luteolin, baicalein), 2 polymethoxyflavones (tangeritin, 5,6,7,4'-tetramethoxyflavone), and 4 isoflavonoids (daidzein, genistin, ononin, puerarin) against *E. coli* and found that the antibacterial activity decreased in the following order: flavonoids > polymethoxyflavones > isoflavonoids. They also found that flavonoids rigidified the liposomal membrane, while the other compounds increased membrane fluidity. They suggested that the interaction of gallic and ferulic acids with bacterial cytoplasmic membranes causes hydrophobic changes, a decrease in negative surface charge, and local rupture and pore formation with leakage of intracellular constituents. The studies in the last decade have shown that phenolics with an OH group at the position 3 in the C-ring decrease the mem-

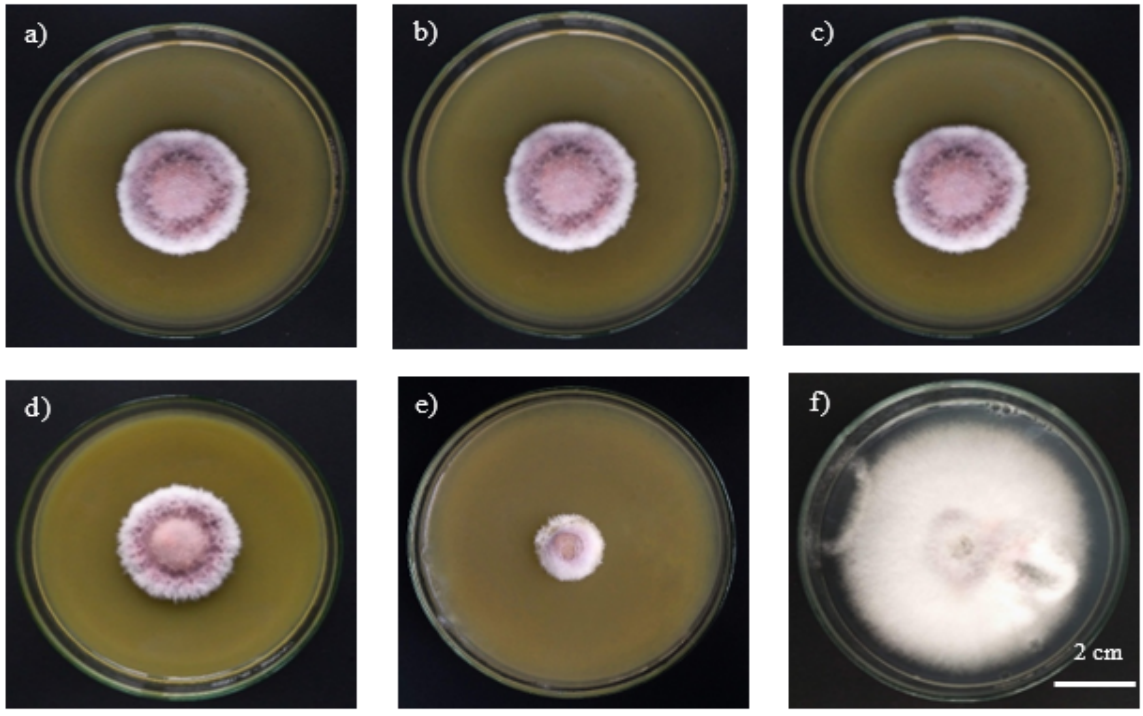

Figure 3. The antifungal ability of *C. odorata* extract against *F. oxysporum* at different concentration (a) 0.1% v/v; (b) 0.2% v/v; (c) 0.3%; (d) 0.4% v/v; (e) 0.5% v/v; (f) Negative Control.

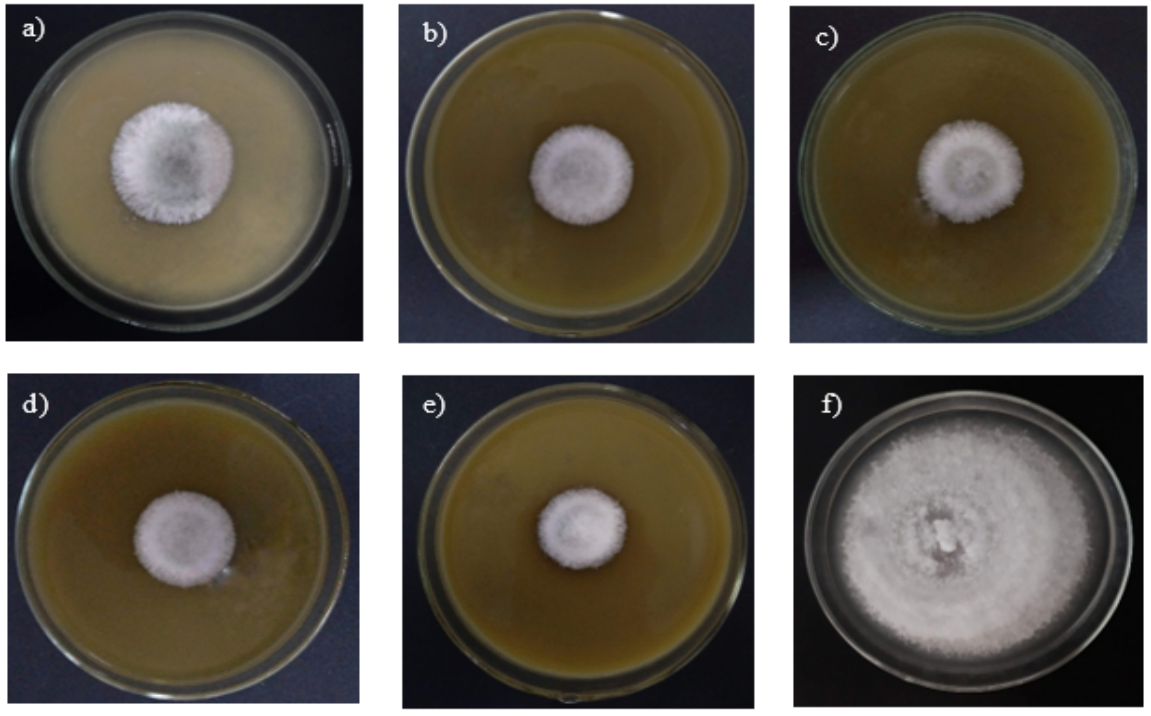

Figure 4. The antifungal ability of *C. odorata* extract against *C. sianmense* at different concentration (a) 0.1% v/v; (b) 0.2% v/v; (c) 0.3%; (d) 0.4% v/v; (e) 0.5% v/v; (f) Negative Control.

brane fluidity leading to membrane rigidification (Tsuchiya, 2010; Ting, 2013). Many studies have used membrane models to elucidate the interaction of polyphenols with the phospholipids of the bilayer lipid membrane. In addition, *F. oxysporum* YT1 and PT1 were cultured in vitro in the presence of *C. odorata* leaf extract. Mycelium growth was reduced at concentrations of 20, 30 and 40 g/L but stopped at 50 g/L. (Kra, 2009). The above was resulted contribute to affirm that *C. odorata* leaves extract has good antifungal ability as reported by Adeyemo (2018) using *C. odorata* extract by maceration to inhibit *P. megakarya* from 15.00 mm to 32.00 mm at concentrations of 1% and 5% respectively for positive control 41.00 mm.

4. Conclusions

Optimal total phenolic compounds TPC of *C. odorata* leaves was 75.76 mgGAE/g at 62°C for 3 min with solvent to feed ratio of 14:1 (mL/g) and ethanol concentration of 50% (v/v). Ethanol extract clearly showed antifungal activity through antifungal index. The highest antifungal effect on *P. capsici*, *F. oxysporum* and *C. siamense* were 79.65%, 78.66% and 78.42% in 0.5% of ethanol extract, respectively. The findings demonstrated the potential of using *C. odorata* leaf extract as a source of biopesticide production, not only the environment but also the ecosystem and biodiversity.

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Conflict of interest declaration

The authors have declared no conflict of interest.

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Spray-drying microencapsulation of β-carotene by polysaccharide from yeast cell walls

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ABSTRACT

It is well known that β-carotene plays an important role in human health. However, it is susceptibly degraded by environmental conditions including temperature, light and oxygen, due to its natural structure of conjugated double bonds chain. Polysaccharide (PS) from the yeast cell walls has appeared to be an excellent choice of encapsulating agent that can be used to encapsulate biologically active substances . Objective of this study was to investigate the effects of PS concentration (20, 30, 40 and 50%) compared to MD, high pressure homogenization time (5, 10, 15, 20 min) and inlet air temperature of spray drying (130, 140, 150, 160°C) on microencapsulation yield (MEY), microencapsulation efficiency (MEE), moisture content (MC) and antioxidant capacity. The results showed that the highest values of MEY (354.4 µg/g) and MEE (90.2%), the low value of MC (7.0%) and antioxidant capacity (69.07%) were successfully obtained at PS concentration of 30% (w/v), homogenization of 15 min and inlet air temperature of 150°C. Under those conditions, the encapsulated β-carotene powder was examined by scanning electron microscopy (SEM) and it is confirmed that the micro-particles had various sizes which are a typical characteristics of spray dried powders, spherical shapes and were free of cracks and pores. As a result, it can be concluded that β-carotene was successfully encapsulated in the PS and MD matrix and could then be easily incorporated into various foods.

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1. Introduction

Brewer's yeast is a by-product of brewery, of which a small portion is sold to livestock households for use as direct feed, and the rest is discarded into the environment or need to be treated at high cost. However, the composition of the yeast by-product contains a large amount of yeast cells, in which cell walls account for about 20-30% of dry biomass. The main components in the yeast cell wall are polysaccharides (PS), which include mannoproteins and β-glucan, accounting for 30-40% and 50-60%, respectively, and a very small amount of chitin (Kogan & Kocher, 2007). As a result, it is important to utilize the yeast by-product for food ingredient applications.

Microencapsulation of bioactive substances is one of the most effective methods applied in the food industry. Currently, there are various wall materials used for microencapsulation such as maltodextrin (MD), whey protein and gum. PS from yeast cells has been known as alternative wall material that can be used to encapsulate bioactive components for maintaining the stability of the bioactive compounds from sensitive environmental conditions such as temperature, pH,

light, and oxygen. Shi et al. (2008) studied using *Saccharomyces cerevisiae*'s yeast cells to encapsulate resveratrol compounds. The results showed that after being absorbed into the yeast cell wall, resveratrol compound improved water solubility and chemical structure was kept stable and the biological activity was ensured. The research of Czerniak et al. (2015) on using yeast cells to encapsulate fish oil products showed that the microencapsulation could help products withstand the high temperatures during the drying process and prevent the oxygen diffusion from products and limit the oxidation of fish. Similarly, Sultana et al. (2018) reported that yeast cell walls can be effectively used to microencapsulate d-limonene essential oil. After the microencapsulation, the essential oil was more durable to temperature, which could be convenient for spray-drying to improve the quality of the resultant product from oxidation.

β-carotene is a food colorant and a highly bioactive substance which has been widely used in food industry. However, it is unstable when exposed to light, oxygen and temperature. Thus, encapsulation of β-carotene has been reported to effectively protect against the effects of the susceptible environment conditions. Loksuwan (2007) stated that β-carotene could be effectively encapsulated using spray drying. Kha et al. (2010) carried out the encapsulation of carotenoid compounds in Gac fruit (*Momordica cochinchinensis*) by spray-drying method with maltodextrin. The results indicated that the spray drying temperature significantly affected the reduction of carotenoids in the samples. If the drying temperature increased from 120 to 200°C antioxidant activity decreased from 0.14 to 0.08 mmol TE/g powder and the microencapsulation efficiency reduced from 76.6% to 48.0%. Another study of Pham-Hoang et al. (2018) reported that using yeast cells *Yarrowia lipolytica* to microencapsulate β-carotene demonstrated the highest encapsulation efficiency. In addition, the use of ultrasound assistance was reported to significantly improve the carotene encapsulation efficiency.

There is lack of information on encapsulation of β-carotene using PS as wall material in the published literature. Therefore, this study is aimed to investigate effects of PS concentration, homogenization time and inlet air temperature of spray drying on microencapsulation yield (MEY) and microencapsulation efficiency (MEE). In addition, physical properties and antioxidant capacity of the encapsulated β-carotene powder were also evaluated and compared.

2. Materials and Methods

2.1. Materials

β-carotene (97%) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sigma-Aldrich Pty. Ltd. All solvents used in this study were of analytical grade. PS was extracted from yeast by-product obtained from Saigon Brewery, Vietnam by combining ultrasonic and enzyme methods. Briefly, yeast by-product in the gelatious form was mixed with distilled water at ratio of 1:3 (w/w), respectively, allowed to settle for 1 h and then decanted the upper layer and water. This step was performed in triplicate. The collected yeast residue (about 10 g) was added 3% (v/w) protease enzyme and incubated at 45°C for 6 h. Afterward, water (40 mL) was added, ultrasound treated for 10 min and centrifuged at 4500 rpm for 15 min. The collected residue was washed with water three times, and centrifuged at 4500 rpm for 15 min to collect PS.

2.2. Microencapsulation of β-carotene

For core material, β-carotene was first dissolved and mixed thoroughly in the solution containing alcohol and acetone at ratio of 1:1 (w/w). For wall material preparation, maltodextrin (10 g) mixed with polysaccharide at different concentrations of 20, 30, 40 and 50% (w/v), and then the mixture was dissolved in 50 mL of 0.37% trehalose solution. For comparison, maltodextrin used as wall material was also prepared.

To create emulsions, the β-carotene solution (10 mL) was added dropwise to wall material solutions (50 mL) while mixing using homogenizer at 4200 rpm for different times (5, 10, 15 and 20 min) to allow full incorporation. The stable solutions were spray-dried in a LabPlant SD-06 spray dryer (LabPlant UK Ltd., North Yorkshire, UK). The dryer was equipped with a two-fluid nozzle atomizer (0.5 mm diameter). The operating conditions of the spray drying were inlet temperatures of 130, 140, 150 and 160°C, outlet temperatures of 73-80°C and pressure of 2 bar, and the feed flow rate was about 200 mL/h. The obtained encapsulated powder was recovered from the collecting chamber. The powders were stored at 4°C

in a vacuum bag until analysis (within 24 h). The sample preparation was done in triplicate.

For stability test, after microencapsulation of β-carotene by spray drying, the encapsulated β-carotene powder was stored at different storage conditions including exposure to light and oxygen. The vacuum packaged powder (considered as relatively without air) was used as control. The external morphography of powder particles using scanning electron microscopy (SEM) and antioxidant capacity at different times of 4 and 8 h were evaluated. The commercial β-carotene powder was also used for comparison in terms of antioxidant capacity.

2.3. Analytical methods

2.3.1. Microencapsulation Yield (MEY)

The MEY was calculated as the amount of microencapsulated β-carotene in 1 g of spray dried powder (Paramera et al., 2011).

$$\text{MEY} = \frac{\text{Amount of microencapsulated } \beta\text{-carotene}}{\text{Amount of spray dried powder}} \ (\mu g/g)$$

β-carotene content determination: The content of β-carotene in the samples was determined by UV-Vis method by measuring absorbance at 450 nm wavelength and calculated according to Beer-Lambert law (Rodriguez-Amaya, 2001).

$$\beta\text{-carotene content} = \frac{\text{Abs at } 450 \times y \times 1000}{2620 \times 100} \ (mg)$$

Where:

y (mL) is the total volume of measured solution

2620 is the molecular absorption factor

1000 is the conversion factor from g to mg

2.3.2. Microencapsulation efficiency (MEE)

The MEE was calculated as the percentage between the amount of microencapsulated β-carotene and the initial amount of β-carotene (Pham-Hoang et al., 2018).

$$\text{MEE} = \frac{\text{Amount of microencapsulated } \beta\text{-carotene}}{\text{Initial amount of } \beta\text{-carotene}} \times 100 \ (\%)$$

Microencapsulated β-carotene content determination: Weighing 1 g of spray-dried powder and dissolving in 15 mL hexane, and then vortexing for 5 min, and then centrifuging at 4500 rpm for 5 min, and finally obtaining the supernatant. The supernatant was measured at 450 nm wavelength to determine the surface β-carotene content. The blank sample was hexane.

Amount of microencapsulated β-carotene = (Initial amount of β-carotene) - (Amount of surface β-carotene).

Determination of antioxidant capacity by DPPH method: According to Brand-Williams et al. (1995), DPPH was diluted at a concentration of 40 µg/mL with methanol. For the commercial carotene sample, the sample was dissolved to 0.15 mg/mL with hexane. About 2 mL of supernatant was added with 2 mL of DPPH solution, incubated for 30 min and finally determined the antioxidation capacity of the sample at 517 nm wavelength.

For the spray dried powder sample, the sample was dissolved in water, centrifuged at 5000 rpm for 5 min, and then collected the residue. The residue was continually dissolved with 10 mL of 1M buffer solution of sodium acetate for 20 min. Next, about 10 mL of hexane added to obtain β-carotene concentration of 0.15 mg/mL, then 2 mL DPPH was added. The samples were incubated at ambient temperature for 30 min and measured at 517 nm wavelength.

The percentage of free radical reduction of the sample was calculated as follows:

$$I(\%) = 100 \times \frac{A_0 - A_1}{A_0}$$

Where:

I (%) is the percentage of free radical reduction of the sample at 517 nm

A_0: Absorbance of DPPH

A_1: Absorbance of the tested sample

The blank sample is a methanol solution

2.3.3. Statistical analysis

The independent experiments and subsequent measurements were done in triplicate. The results were presented as mean values with standard deviations. A one-way analysis of variance (including PS concentration, homogenization time and inlet air temperature) and LSD (least significant difference) were used to analyze the data using the Statgraphics centurion XVI software.

3. Results and Discussion

3.1. The effect of wall material on microencapsulation process

Carrier (wall material) is an important ingredient affecting the microencapsulation process, including MEE and MEY. One of the most important characteristics of wall material includes non-reactive with the core, ability to encapsulate

Table 1. The effect of polysaccharides (PS) content combined with maltodextrin on microencapsulation process

PS content (%, w/v)	Amount of microencapsulated β-carotene (mg)	Microencapsulation Yield (µg/g)	Microencapsulation efficiency (%)
20	2.958 ± 0.01^{a}	197.60 ± 0.07^{b}	75.65 ± 0.92^{a}
30	3.293 ± 0.01^{b}	205.88 ± 1.27^{b}	81.30 ± 7.72^{b}
40	3.289 ± 0.02^{b}	173.95 ± 0.64^{a}	$80{,}55 \pm 8.55^{b}$
50	3.280 ± 0.02^{b}	171.16 ± 0.64^{a}	79.95 ± 1.92^{b}

The values in the same column followed by different superscripts (a-b) were significantly different ($P < 0.05$).

and maintain the core inside the microcapsule; and ability to provide maximum protection to the core against adverse conditions (Gharsallaoui et al., 2007; Nazzaro et al., 2012). It is highly desirable to achieve the highest ratio of the core (bioactive compounds) to the wall material. The concentration of the wall material was too high leading to an increase in the viscosity and difficult to spray-dry (Tonon et al., 2008). In this experiment used PS 20, 30, 40 and 50% (w/v) combined with MD to microencapsulate β-carotene by spray drying, inlet air temperature 150°C, feed rate 200 mL/h, and pressure of 2 bar. The effect of PS concentration on MEE and MEY is shown in Table 1.

According to Table 1, the amount of PS being 20% was not enough to encapsulate all the β-carotene, indicated by low MEE. The largest amount of microencapsulated β-carotene and the highest MEE were 3.29 mg and 81.30% respectively, when incorporated with 30% of PS. When increase in PS amount, up to 40% or 50% PS resulted in insignificantly decrease in the amount of β-carotene. It can be concluded that high content of PS of 30% could be enough for encapsulating β-carotene.

Wagner & Warthesen (1995) performed the microencapsulation of carotene with MD powder. The results showed that about 38% of β-carotene was not encapsulated. Similarly, Loksuwan (2007) used MD by spray drying method to microencapsulate β-carotene and reported that the total amount of β-carotene accounted for 46.74% of the total weight of spray dried powder. It can be seen that only MD used as wall material was not effective, there is a need to combine with other wall materials. Sultana et al. (2018) encapsulated d-limonene by spray-drying method with MD and yeast cell walls and the results showed that about 82% of the d-limonene was retained in the MD and sodium caseinate matrix and 26% was retained in the yeast cell wall.

For comparison (Figure 1), the microencapsulation of β-carotene powder using PS and MD mixture as wall material had MEE reached up to 87.15%, which was 1.5 times higher than that of powder using only MD (56.90%). Furthermore, a higher moisture content of microencapsulated sample using only MD (8.3%) was observed as compared to the ones using the mixture of PS combined with MD (MC of 7%), resulting higher MY, which were 52.97% and 45.20%, respectively. It is well known that higher moisture content could result in instability during storage.

It can be concluded that the mixture of PS and MD has shown excellent property of wall material for encapsulation of β-carotene. The microencapsulated β-carotene powder has ability to withstand the impacts of light and oxygen better than that of the free β-carotene form (without wall material). The reason is that the wall material containing PS (β-glucan) could protect β-carotene and has a good antioxidant capacity.

According to Sun-Waterhouse et al. (2011), an appropriate selection of wall materials is very important. Generally, combinations of various encapsulating agents are required to effectively protect and control the bioactive compounds. Thus, combination of PS and MD to microencapsulate β-carotene, resulted in higher MEE than only MD. It is highly recommended that 30% PS in combination with MD (10 g) should be used to encapsulate β-carotene.

3.2. The effect of homogenization time on the β-carotene microencapsulation process

The homogenization has positively influenced stability of β-glucan in the solution and prevented the accumulation of β-glucan (Bzducha-Wróbel et al., 2014), and also reduced the size of the emulsion droplets. As such, the increase in the

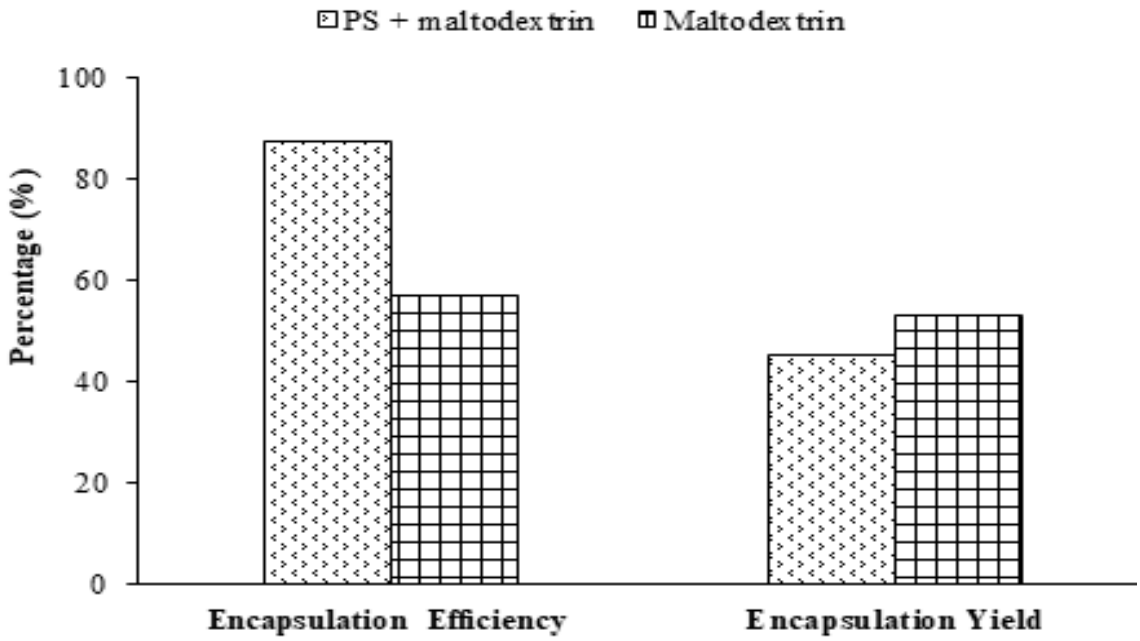

Figure 1. Microencapsulation efficiency and Microencapsulation yield of β-carotene using maltodextrin (MD) and the mixture of polysaccharides and MD.

Table 2. The effect of homogenization time on the β-carotene microencapsulation process

Homogenization time (min)	Amount of β-carotene microencapsulated (mg)	Microencapsulation Yield (µg/g)	Microencapsulation Efficiency (%)
5	4.658 ± 0.47^{b}	226.0 ± 22.63^{b}	78.2 ± 7.85^{b}
10	5.251 ± 0.06^{c}	251.0 ± 2.83^{c}	88.1 ± 1.13^{bc}
15	5.421 ± 0.04^{c}	268.5 ± 2.12^{c}	91.0 ± 0.71^{c}
20	3.910 ± 0.07^{a}	188.5 ± 3.54^{a}	65.6 ± 1.13^{a}

The values in the same column followed by different superscripts (a-c) were significantly different ($P < 0.05$).

contact area between the wall material and the carotene as well as the mass transfer and evaporation rate during the atomization are achievable (Shappley et al., 1998). As a result, higher MEY and MEE could be obtained. In this experiment, the most suitable homogenization time (5, 10, 15 and 20 min) was tested and the results are shown in Table 2.

When the homogenization time increased from 5 to 10 min, the amount of microencapsulated β-carotene increased from 4.658 mg to 5.251 mg. However, the amount of β-carotene in the encapsulated powder had no statistically significant difference when increasing the homogenization time from 10 to 15 min and even reduces to 3.910 mg when the initial solution was homogenized for 20 min. The highest MEYs were obtained for the homogenization times of 10 and 15 min, being 251.0 and 268.5 µg/g, respectively. At 20 min, the MEY was only 188.5 µg/g. The highest MEEs were found to be 88.1% and 91% corresponding to the homogenization times of 10 and 15 min, respectively. At 20 min, the MEE decreased due to longer homogenization time, it may negatively affect the structure of the wall material (PS), leading to inefficient microencapsulation.

Trehalose is considered to be a polysaccharide protective agent against environmental stress conditions, keeping its structure from being changed during homogenization process. Trehalose also has another role in the better transmembrane transfer of carotene (Golovina et al., 2010). Thus, the homogenization time of 15 min for initial solution before spray drying should be carried out.

Table 3. Effect of inlet spray drying temperature

Inlet temperature (°C)	Amount of microencapsulated β-carotene (mg)	Microencapsulation Yield (µg/g)	Microencapsulation Efficiency (%)
130	3.495 ± 0.06^{b}	318.40 ± 27.55^{ab}	79.7 ± 1.27^{b}
140	3.764 ± 0.06^{c}	327.63 ± 31.22^{ab}	86.6 ± 2.47^{c}
150	3.955 ± 0.07^{d}	354.36 ± 2.16^{b}	90.2 ± 1.38^{c}
160	3.240 ± 0.95^{a}	267.02 ± 35.58^{a}	73.9 ± 2.12^{a}

The values in the same column followed by different superscripts (a-c) were significantly different ($P < 0.05$).

3.3. Effect of inlet temperature on the β-carotene microencapsulation process

The inlet temperature affects the water evaporation rate and film formation. When temperature is too low, the moisture of product is still high, so the particles easily stick to the drying chamber wall, resulting in decreasing efficiency. At higher inlet temperatures, the evaporation rate of water on the surface of the spray-dried powder is also higher, leading to a better formation of the film, so locked up well and higher protection of bioactive compounds, as a result, MEY and MEE are improved. However, when drying temperature is too high, it can flaw on the film. Thus, it reduces biological activity and loss of MEY and MEE (Kha et al., 2014). It is desirable to determine the most suitable spray drying temperature, and the effect of inlet temperature on the MEY and MEE is presented in Table 3.

Table 3 shows that when the inlet temperature increased from 130°C to 150°C, the amount of microencapsulated β-carotene increased gradually for up to 12%. The reason is higher temperature resulted in better film formation, which led to the increases in MEY and MEE. However, at 160°C, the amount of microencapsulated β-carotene significantly decreased from 3.955 mg to 3.240 mg, indicated by lower MEE and MEY.

According to Shu et al. (2006), high inlet temperature could disrupt the balance between water evaporation rate and film formation process, resulting in the disrupted membrane system of the microcapsules, and reducing the retention of β-carotene in the microcapsules. As a result, the MEY and MEE were decreased. Similar result was found in the report of Kha et al. (2010) who performed the encapsulation of carotenoid compounds in Gac fruit by spray drying method with MD. The results showed that the inlet temperature also affected the reduction of carotenoids in the samples. An increasing the inlet temperature from 120°C to 200°C resulted in a decrease in encapsulation efficiency and antioxidant capacity from 76.6% to 48.0% and 0.14 to 0.08 mmol TE/g powder, respectively.

It can be concluded that inlet temperature is the most important parameter that could affect the MEY and MEE. For this study, the inlet temperature of 150°C should be chosen for better retention of β-carotene in the microencapsulated powder.

3.4. Antioxidant capacity of the powder samples

In this study, antioxidant capacity of the commercial β-carotene and microencapsulated β-carotene powders under light and oxygen conditions was compared at different times (Figures 2 and 3). The antioxidant capacity of the commercial sample decreased sharply from 75.66% to 40.61% for the first 4 h (a decrease of about 35%) and continued to decrease to 35.91% for the next 4 h. However, the antioxidant capacity of the microencapsulated β-carotene sample decreased from 69.07% to 55.08% (a decrease of about 12%) for the first 4 h, and the antioxidant capacity decreased slightly for the next 4 h. Thus, it can be concluded that the effect of light on the microencapsulated β-carotene powder was much lower than that of the commercial carotene sample.

Similar to the light, when exposed to oxygen, the antioxidant capacity of the commercial sample decreased sharply from 75.66% to 47.08% for the first 4 h (a decrease of about 28.58%) and continued to decrease to 44.02% for the next 4 h. However, the antioxidant capacity of the microencapsulated β-carotene samples decreased from 69.07% to 59.50% (a decrease of 9.57%) for the first 4 h of exposure to oxygen, and the antioxidant capacity decreased slightly for the next 4 h. Thus, it can be clearly seen that the effect

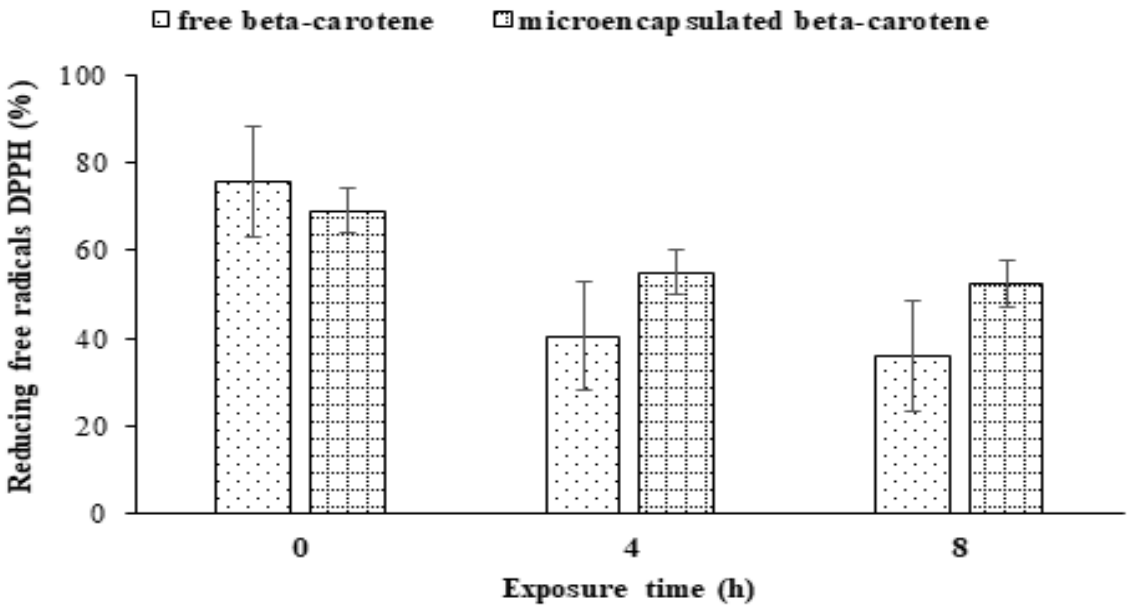

Figure 2. The effect of light on the antioxidant capacity of β-carotene.

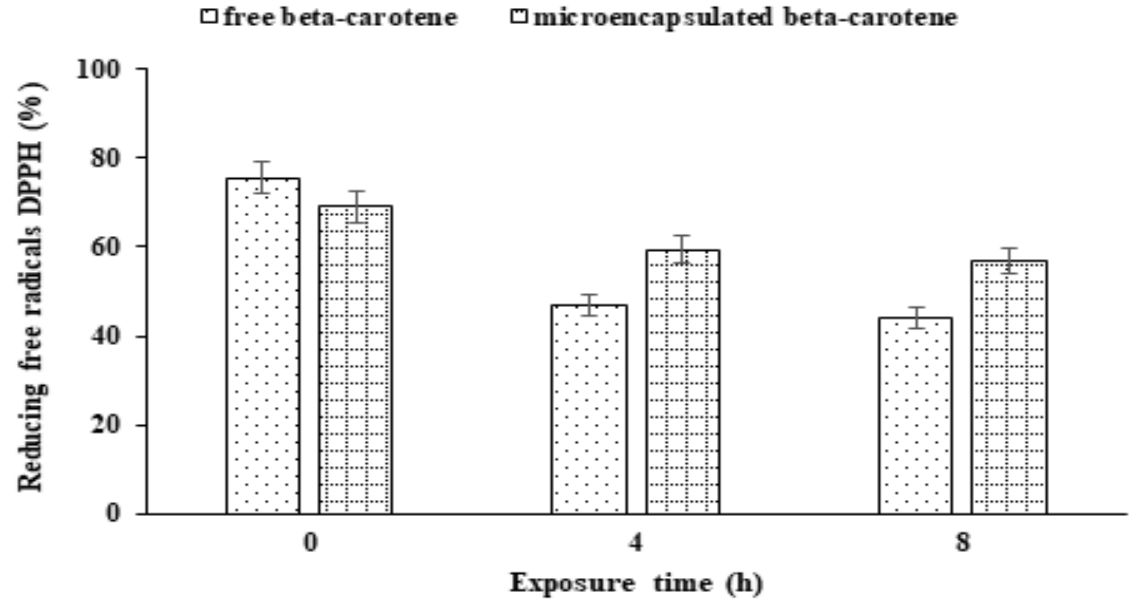

Figure 3. The effect of oxygen on the antioxidant capacity of microencapsulated β-carotene powder.

of oxygen on microencapsulated carotene in powder samples was lower than that of commercial carotene samples.

3.5. Microstructure of the microencapsulated β-carotene powder

The color of the spray-dried powder was off-white. The microstructure of the encapsulated β-carotene powders observed using SEM is presented in Figure 4. The SEM results showed that the microencapsulation of β-carotene with only MD created the spherical particles, including smooth and concave surfaces. In comparison with the microencapsulation of β-carotene with the combination of MD and PS, there were more concave surfaces created. It could be explained that a great pressure during the spray-

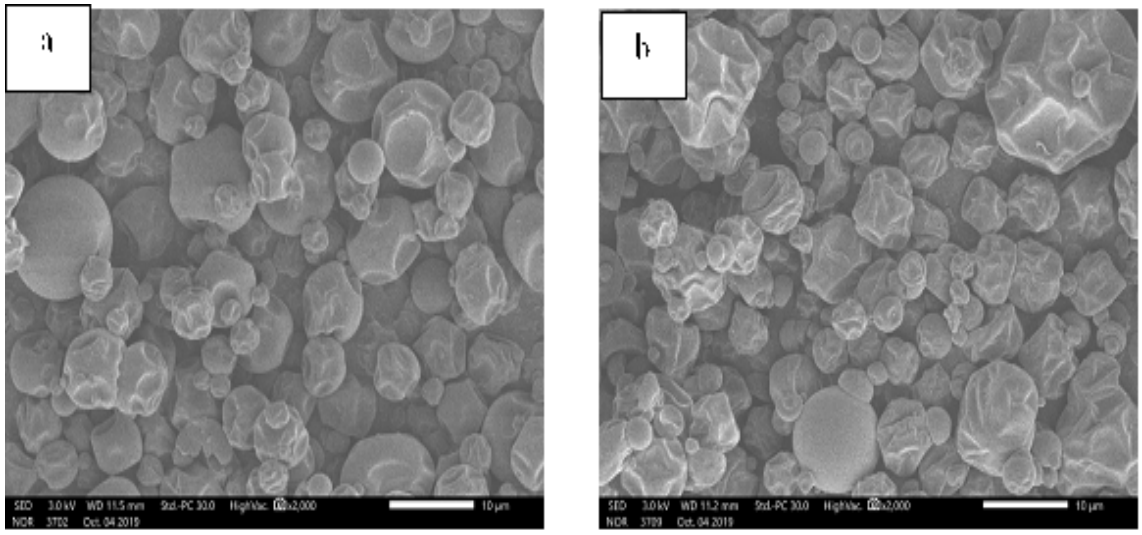

Figure 4. SEM microstructure of spray-dried encapsulated powders (a: maltodextrin, b: maltodextrin + polysaccharides). SEM: Scanning electron microscopy.

drying process could cause the particle's surfaces become convex and concave differently. The sizes of microencapsulated particles were not uniform as well. The SEM results were also quite similar to the study of Loksuwan (2007). The less concave surfaces of MD were created because these types of sugar having low molecular weight could act as a plasticizer to prevent the surface shrinkage during the spray drying process. According to Ye et al. (2000) plastics are very important to the formation of spherical microscopic capsules having smooth surfaces.

4. Conclusions

In conclusion, the effects of wall material (PS and MD) concentration, homogenization time and inlet air temperature of spray drying on the properties of the encapsulated β-carotene powder were investigated. MEY, MEE and antioxidant capacity were significantly affected by those parameters. High MEE in terms of high retention of β-carotene in the powder could be obtained when the initial solution containing PS and MD as wall material was homogenized for 15 min and spray dried at the inlet temperature of 150°C. The encapsulated β-carotene powder was found to be stable under exposure to light and oxygen due to low moisture content and free of cracks and pores as compared to the commercial one. The resultant powder could be then incorporated in to various foods for health benefits.

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Effects of different extraction methods on the recovery yield of bixin from annatto seeds (*Bixa orellana* L.)

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ABSTRACT

Bixin is the a principal color component of annatto pigment which is obtained from the seed coat of *Bixa orellana* L., a tropical shrub. The extraction yield of bixin from annatto seeds using acetone, soybean oil and sodium hydroxide solution with different extraction conditions were investigated in this study. The extraction time, temperature, solid-liquid ratio and light exposure showed significant effects on the bixin yield. The extraction using soybean oil had the lowest bixin yield followed by the extraction using sodium hydroxide solution. The extraction using acetone resulted in the highest extraction yield (68.1%) after only 40 min. The extraction using soybean oil at 100°C led to two-fold bixin yield compared to that operated at 80°C while the mild temperature (50°C) was found to be the most suitable for the extraction using sodium hydroxide solution. The exclusion of light exposure by covering extraction beakers with aluminum foil could significantly improve the bixin extraction yield due to the reduction in bixin degradation. Therefore the extraction using acetone avoiding light exposure is suggested for recovering bixin from annatto seeds.

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1. Introduction

Annatto is a natural food colorant providing yellow to red colors depending on the pigment concentration of the solution. Annatto pigment is obtained from the seed coat of *Bixa orellana* L., a tropical shrub. This tree is native to tropical South America and also being cultivated in many countries of Central America, Africa and South Asia. The fruit of *Bixa orellana* L. consists of a pod covered with spines. The pod is usually divided into two halves inside and contains small seeds. Fruits become mature after pollination 5–6 months. Annatto trees reach maximum yield at 4–12 years old and can remain productive for more than 20 years. Pigment content of the seeds ranges from 1.5 to 4% due to variety, cultivation conditions, and postharvest techniques and quality of annatto seeds can be retained for a long time if they are stored in a cool, dark and

dry place (Bechtold & Mussak, 2009).

Annatto pigment has been used for over 200 years as a natural colorant for foods. Although the major source of annatto seeds is South and Central America, the main production of commercial annatto pigment is Europe and the United States which was primarily used to color butter and cheese. Currently annatto is used for a variety of foods such as meat and fish products, soft drinks, sugar confectionery, margarine, ice cream and soups. Annatto is ranked as the second most economically important natural color in the world and it is the most frequently used natural color in the food industry in the United Kingdom (Green, 1995; Lauro & Francis, 2000; Cannon, 2003).

The red pigment is separated from annatto seeds by different techniques like immersion of seeds in hot vegetable oil, dilute alkaline aqueous solutions or organic solvents. In the traditional food recipes, annatto seeds are heated with cooking oil and then the colored oil is decanted and used for preparing rice, soups, and tortillas. At the industrial production, the seeds are immersed in heated vegetable oil to produce bixin slurry and the filtrate can be marketed as a color for high-fat foods. The outer pigment layer of the seeds can also be extracted by mildly alkaline solution, which is then acid precipitated, filtered and dried to give a granular powder of annatto. Solvent extraction of annatto pigment that can produce 80 - 97% purity annatto colorant has been developed to satisfy the demand of more concentrated annatto color products. Numerous organic solvents such as chlorinated hydrocarbons, mixtures of ethanol and chloroform, acetone, ethanol, ethyl acetate, hexane, methanol or alcoholic sodium hydroxide have been reported to be suitable for producing annatto concentrates.

The principal color component of annatto pigment is bixin which constitutes more than 80% of color content (Preston & Rickard, 1980) of annatto. Bixin is very susceptible with processing conditions such as temperature, light and oxygen which might cause a significant color loss of annatto pigment. A number of studies have focused on the degradation of bixin during processing and storage, and in foods added with annatto pigment. For examples, stability of annatto solution extracted by chloroform to light, air, antioxidants and pro-oxidants has been described by Najar et al. (1988). Light was found as the most destructive agent followed by the pro-oxidant, benzoyl peroxide. Bixin was observed to be more stable at intermediate/higher water activities in a study by Gloria et al. (1995). The effects of processing conditions on the stability of annatto added in to cakes, chegodis, biscuits and fried rice was reported (Prabhakara Rao et al., 2005) and the deep fat fried processing of chegodis caused the maximum loss of bixin (65%). Beside the effect on color intensity of annatto pigment, the decomposition of bixin produces volatile compounds like m-xylene, toluene and toluic acid (McKeown, 1965) which can cause some negative effects to human health.

Although studies related to techniques for recovering bixin from annatto seeds have been reported, a comprehensive research comparing the effectiveness of different extraction methods using various conditions for the recovery of pigment from this material is limited. Thus the extraction yield of bixin, the principal color component of annatto pigment, obtained from various extraction methods and extraction conditions were investigated in the present study.

2. Materials and Methods

2.1. Materials

Annatto seeds were purchased from a local market in the Central Highland of Vietnam. Soybean oil (Ottogi) was purchased from a local market in Gwangju, South Korea.

Acetone and sodium hydroxide were supplied by Dea Jung company and pentane was provided by Kanto Chemical Company (Seoul, South Korea); tetrahydrofuran ($\geq$ 99%), ethylbenzene (99.8%), toluene (99.8%), m-xylene ($\geq$ 99%), and o-xylene ($\geq$ 99%) were obtained from Sigma-Adrich (Darmstadt, Germany). The other reagents were in analytical grade.

2.2. Extraction of total bixin content from annatto seeds

Bixin content in annatto seed was determined according to the method of Balaswamy et al. (2006). Annatto seeds (2 g) were weighed exactly in a beaker wrapped and covered by aluminum foil to prevent the loss of bixin by effects of light. Pigment in the seeds was then extracted repeatedly by chloroform until total pigment is removed from the seeds (determined by observing color of

the extracts). During extraction process, the extracts were collected into a brown glass bottle and stored in a refrigerator. The extract was then diluted and measured absorbance at 487 nm for determining bixin content.

2.3. Extraction of bixin using different solvents

Extraction by soybean oil: 5g of annatto seeds was submerged into a beaker containing soybean oil with stirring at 80°C and 100°C (Preston & Rickard, 1980). Extraction by acetone: 5g of annatto seeds was extracted by acetone in a beaker with stirring at 50°C (Balaswamy et al., 2006).

Extraction by sodium hydroxide solution: 5g of annatto seeds was submerged and stirred in a beaker containing NaOH 0.1N solution at 40, 50 and 60°C (Shuhama et al., 2003).

Extraction of bixin from annatto seed in darkness (exclusion of light): Annatto seeds were submerged and stirred in a beaker which was wrapped and covered by aluminum foil to exclude light exposure.

2.4. Analysis of bixin content in annatto extracts

The bixin content in the extracts was determined by measuring absorbance solutions at 487 nm using a spectrophotometer (Optizen 2120UV, Mecasys Co.Ltd, Daejeon, South Korea) (Smith, 2006). One milliliter of bixin extract was dissolved in 10 mL tetrahyrofuran in 100 mL-volumetric flask and then this solution was diluted by acetone to obtain a solution with absorbance ranged from 0.2 to 1.0. Absorbance of the diluted solution was measured in 1 cm cuvet at 487 nm. The concentration of bixin in solution was calculated based on the extinction coefficient ($E_{1\%}^{1cm}$) of 3.090, according to following formula:

$$\text{Bixin concentration (\%)} = \frac{A}{3.090} \times \frac{100,000}{\text{sample weight (mg)}} \times 100$$

2.5. Data analysis

All experiments were performed in triplicates, and the results were expressed as the mean ± standard deviation. LSD tests were used for comparisons amongst values. Differences were considered to be significantly different at $P < 0.05$.

3. Results and Discussion

3.1. Extraction of bixin using sodium hydroxide solution

3.1.1. Effects of temperature

Influences of temperature (40, 50, and 60°C) on the yield of bixin extracted from annatto seeds by sodium hydroxide solution is shown in Figure 1. Temperature showed significant effects on bixin yield at the beginning of the processes. Then only significantly lower yields were observed in the extraction at 40°C as compared to that at other temperatures. No significant differences in bixin yield appeared between the processes carried out at 50 and 60°C. At the extraction time of 100 min, the yield of bixin seemed to reach the plateau when there were no significant changes in the yield compared to that at the extraction time of 80 min for extractions at 50°C and 60°C. Besides, bixin yield of extraction at 40°C also achieved a similar level with these processes. These results suggested that it is better to carry out the extraction of annatto pigment by sodium hydroxide solution at 50°C because the extraction at this temperature can achieve significantly higher bixin extraction yield than that of extraction at 40°C. Furthermore, this extraction can also reduce the loss of bixin caused by high temperature. The significant effect of temperature on the degradation of bixin was reported in a number of previous studies such as Shuhama et al. (2003), Balaswamy et al. (2006) and Silva et al. (2007).

3.1.2. Effects of solid-liquid ratio on bixin extraction yield

The difference in volume of sodium hydroxide solution had no significant effects on the extraction yield of bixin from annatto seed at the extraction time of 20 and 40 min (Figure 2). However, at 60 minutes of the process, the high volume of sodium hydroxide solution (ratio of 1:10 (g/mL)) showed a significantly higher extraction yield compared to the other solid-liquid ratios. The 60 min extraction at this ratio obtained 7% higher bixin yield than that at 40 min (from 29.7% to 36.7%), whereas the ratios of 1:5 and 1:2 (g/mL) had no significant differences in bixin yield with only small increases of 6 and 3.9%, respectively. Therefore, if the extraction is oper-

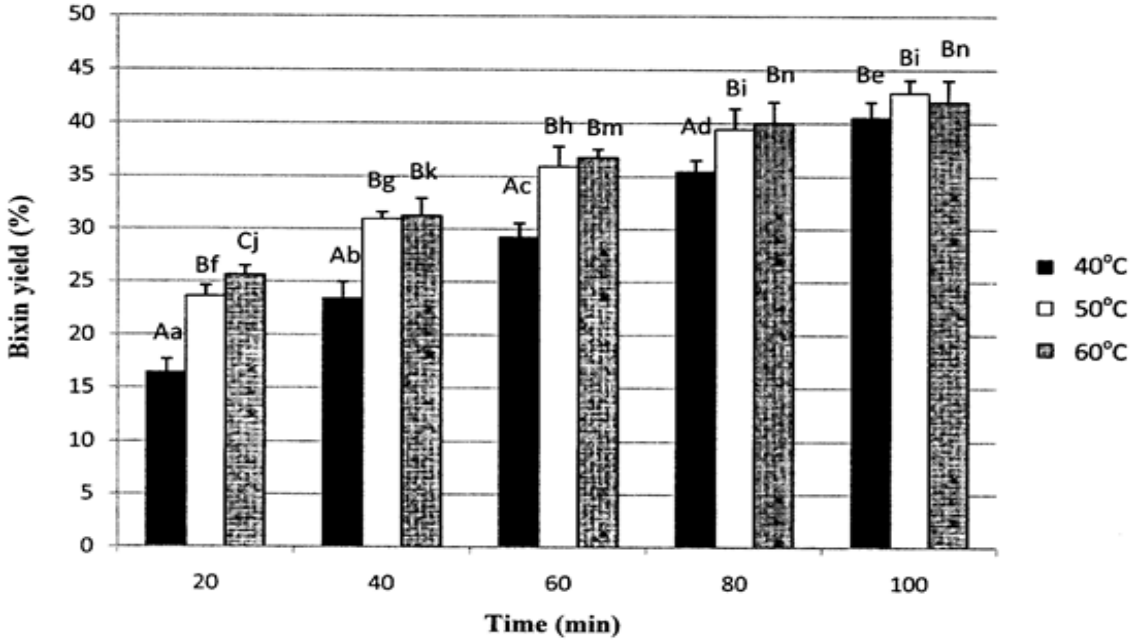

Figure 1. Extraction yield of bixin at different temperatures by sodium hydroxide solution.
Different capital letters show significant differences in bixin yield among different temperatures at the same time ($P < 0.05$).
Different small letters show significant differences in bixin yield among different time intervals of each extraction process ($P < 0.05$).

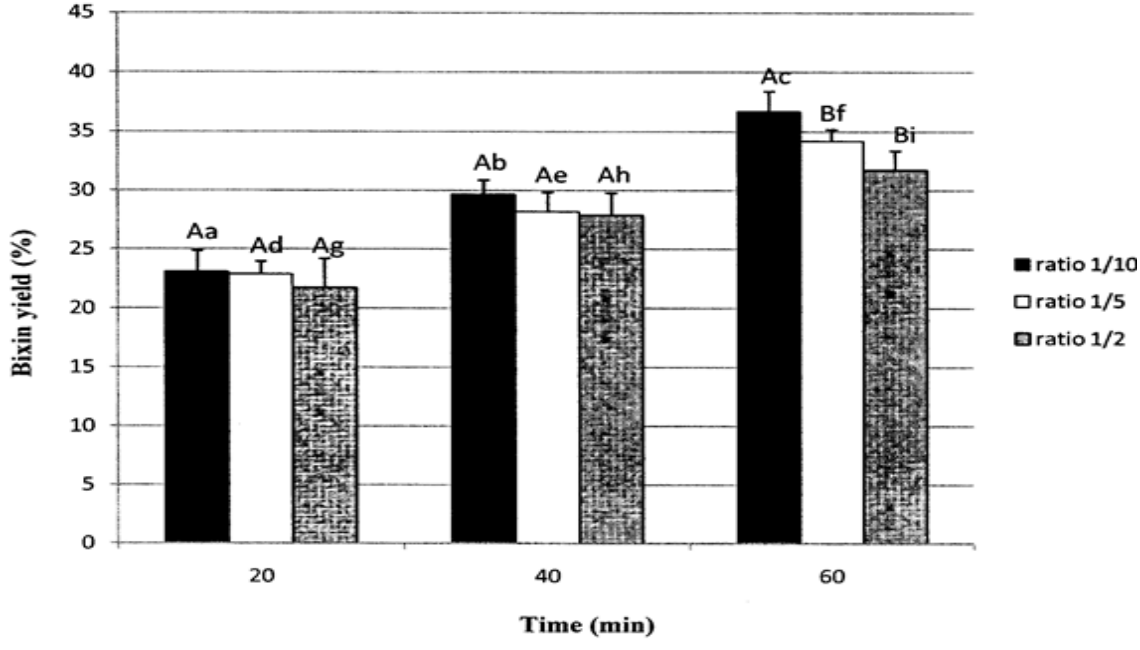

Figure 2. Extraction yield of bixin using sodium hydroxide solution with different solid-liquid ratio.
Different capital letters show significant differences in bixin yield among different solid-liquid ratios at the same time ($P < 0.05$).
Different small letters show significant differences in bixin yield between different times of each extraction using a solid-liquid ratio ($P < 0.05$).

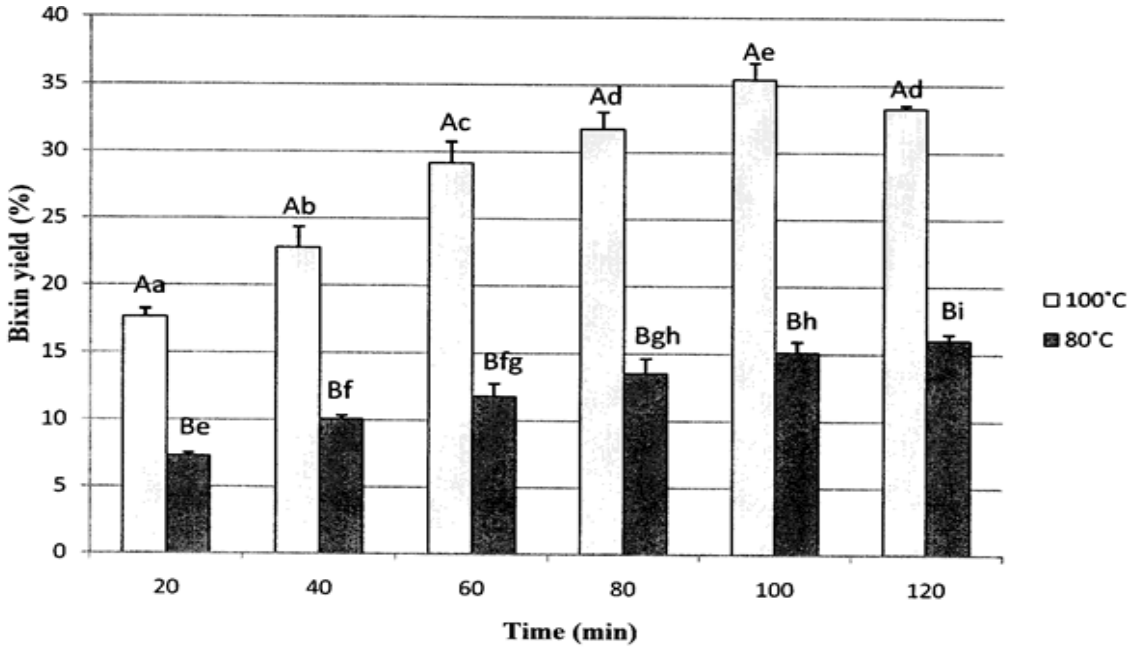

Figure 3. Extraction yield of bixin by soybean oil at 80°C and 100°C.
Different capital letters show significant differences in bixin yield between extraction temperatures at the same time ($P < 0.05$).
Different small letters show significant differences in bixin yield among different times using an extraction temperature ($P < 0.05$).

ated within 40 min, the volume of solution could be considerably reduced without any decrease in bixin extraction yield. This reduction can resulted in a significant energy saving for evaporation of water to produce commercial annatto powder from aqueous alkaline solution of annatto (Shuhama et al., 2003).

3.2. Extraction of bixin using soybean oil

3.2.1. Effects of temperature on extraction yield of bixin

Extraction temperature showed significant influences on the bixin yield in the extraction method using soybean oil (Figure 3). When temperature dropped only 20°C (from 100°C to 80°C), the extraction yields decreased extensively. The extraction at 100°C achieved more than twice bixin yield compared to that of the extraction at 80°C throughout 120 min of extraction time. The increases in bixin yield after every 20 min at this temperature were also significantly higher than those of 80°C extraction. The low bixin extraction yield achieved by soybean oil may not be caused by the solubility of this compound, but because of the severe degradation at high temperature. Consequently, it is better to improve bixin extraction yield in this method by reducing bixin loss than by increasing its solubility based on the increase of soybean oil temperature. These results suggested that extraction at 100°C may be a good option, which not only improve yield of bixin but also reduce the content of undesirable compounds caused by bixin degradation in the extracts.

3.2.2. Effects of solid-liquid ratio and light on extraction yield of bixin

Figure 4 shows bixin yields extracted by soybean oil at the ratios 1:4 and 1:2 (g/mL) and yield of bixin extracted in the darkness at the ratio of 1:2 (g/mL). Surprisingly, the extraction yield derived from the lower ratio (1:2) was significantly higher than that of the higher ratio during 60 min of extraction. This result may be explained by the lower decomposition of bixin in the higher concentrated solutions, which were obtained from lower ratio of seed weight per soybean oil volume. As expected, the extraction in the darkness led to a greater extraction yield of bixin compared to

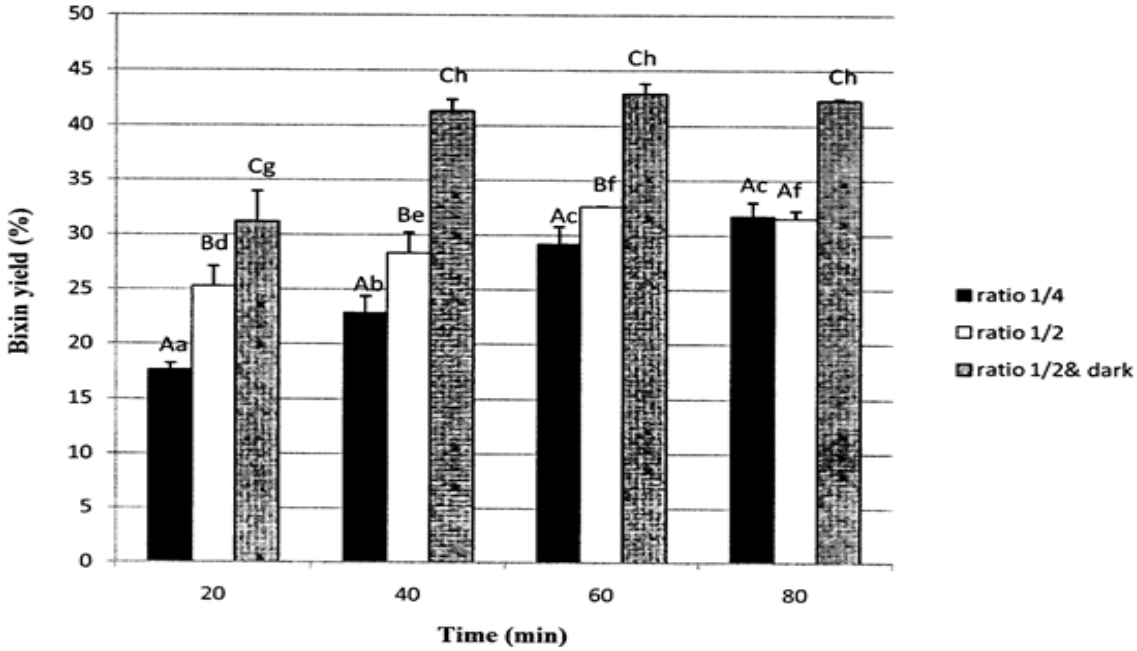

Figure 4. Extraction yield of bixin using soybean oil with different solid-liquid ratio and exclusion of daylight
Different capital letters show significant differences in bixin yield between extraction conditions at the same time ($P < 0.05$).
Different small letters show significant differences in bixin yield between different times of an extraction condition ($P < 0.05$).

the extraction exposed to daylight. Furthermore, this process reached the maximum yield (41.3%) after only 40 min of extraction. This greater extraction yield might be due to the significant decrease in bixin degradation with the absence of daylight, because all other conditions were similar except the exclusion of daylight. These results are in agreements with the previous reports, which showed the predominant impact of light among various factors on the degradation of bixin in annatto colors and foods containing this compound (Najar et al., 1988; Balaswamy et al., 2006).

3.3. Extraction of bixin using acetone

3.3.1. Effects of solvent volume and light to bixin yield extracted by submerging in acetone at 50°C

Bixin yields extracted with different ratios of annatto seed weight per volume of acetone and in darkness 50°C are illustrated in Figure 5. The immersion of annatto seed in acetone for 60 min resulted in a greater amount of extracted bixin compared to the methods using sodium hydroxide solution and soybean oil. This method achieved a yield of 63% at 20 min of extraction while that was lower than 25% in the extraction using the other solvents. The extraction yield obtained by submerging in acetone could reach to the maximum value of 68.1% after only 40 min of the extraction time.

The volume of acetone showed no significant effects on bixin extraction yield at the beginning of process, but the decrease in volume of acetone (ratio of 1:2 g/mL) indicated a considerable decrease in extraction yield at 60 min of extraction time. However, the exclusion of light (extraction in beakers covered by aluminum foil) at this solid-liquid ratio (1:2 g/mL) could improve the bixin extraction yield to a comparable value of that using the ratio of 1:4 (g/mL). Therefore, it seems to have no significant decrease in bixin extraction yield if the ratio of annatto seed weight per acetone volume decreases to 1:2 (g/mL).

4. Conclusions

In this study, the extraction yield of bixin from annatto seeds using acetone, soybean oil and sodium hydroxide solution with different ex-

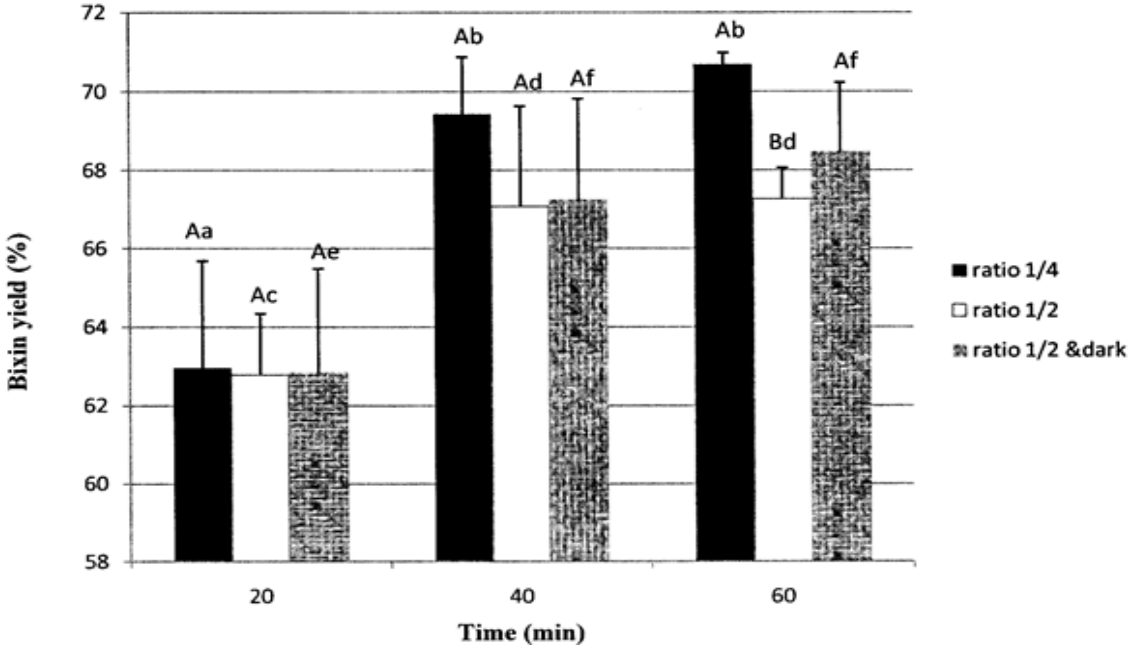

Figure 5. Yield of bixin extracted by submerging in acetone at 50°C with different ratio of annatto seed weight/volume of acetone and in the dark condition.
Different capital letters show significant differences in bixin yield between extraction conditions at the same time ($P < 0.05$).
Different small letters show significant differences in bixin yield between different times of an extraction condition ($P < 0.05$).

traction conditions were investigated. The extraction time, temperature, solid-liquid ratio and light exposure showed significant effects on the bixin yield. The extraction using soybean oil had the lowest bixin yield followed by the extraction using sodium hydroxide solution. The extraction using acetone resulted in the highest extraction yield (68.1%) after only 40 min. The extraction using soybean oil at 100°C led to two-fold bixin yield compared to that operated at 80°C while the mild temperature (50°C) was found to be the most suitable for the extraction using sodium hydroxide solution. The exclusion of light exposure by covering extraction beakers with aluminum foil could significantly improve the bixin extraction yield due to the reduction in bixin degradation. The results suggest that the extraction using acetone with exclusion of light exposure is the most suitable for recovering pigment from annatto seeds.

Conflict of interest declaration

The authors declare no conflict of interest.

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