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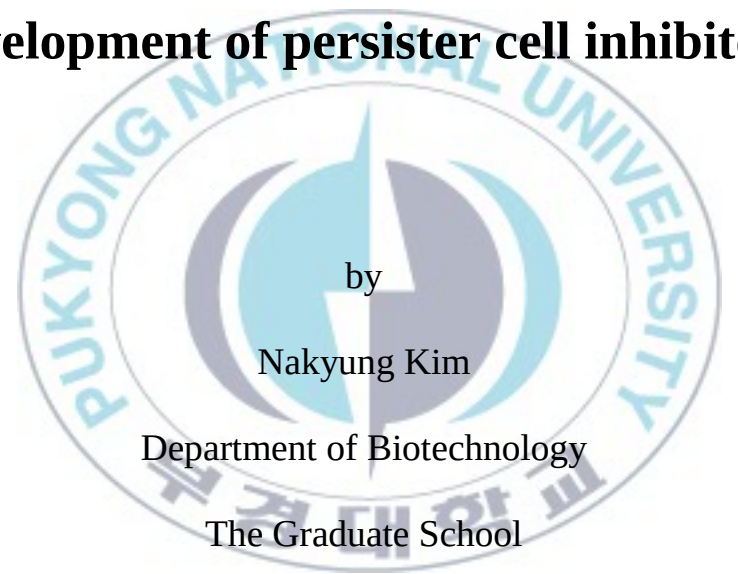
Thesis for the Degree of Master of Engineering

Oil production from microalgae and development of persister cell inhibitors



by

Nakyung Kim

Department of Biotechnology

The Graduate School

Pukyong National University

February 2013

Oil production from microalgae and development of persister cell inhibitors

(미세조류 오일생산과
persister cell 저해 물질 탐색)

Advisor: Prof. Sung-Koo Kim

by

Nakyung Kim

A thesis submitted in partial fulfillment of the requirements
for the degree of

Master of Engineering

In Department of Biotechnology, The Graduate School,
Pukyong National University

February 2013

Oil production from microalgae and development of persister cell inhibitors

A dissertation

by

Nakyung Kim

Approved by:

Joong Kyun Kim
(Chairman)

Gwi-Taek Jeong
(Member)

Sung-Koo Kim
(Member)

February 2013

PUKYONG NATIONAL UNIVERSITY 부경대학교

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미세조류 오일생산과 persister cell 저해 물질 탐색

김 나 경

부 경 대 학 교 대 학 원 생물공학과

요 약

세계는 화석연료의 과다한 사용으로 인한 고갈과 환경오염이라는 커다란 두 가지 문제에 직면하고 있다. 최근 경유 대체연료로 바이오디젤(Biodiesel)이 많은 관심을 받고 있다. 바이오디젤은 기존 경유에 비해 일산화탄소, 미세먼지, 탄화수소, 독성물질 등 대기 오염물질 배출을 크게 줄일 수 있다는 점에서 친환경 자동차 연료로 적합하다. 미세조류 유래 바이오디젤 생산은 다양한 장점을 지니고 있는데 연소에서 나오는 이산화탄소는 다시 광합성 기작에 의해 흡수, 고정되므로 이산화탄소의 순 배출이 거의 없어, 이산화탄소 중립 연료(CO2-neutral fuel)로 큰 주목을 받고 있다. 해양 미세조류는 식량난을 형성하지 않고, 탄소 고정력이 다른 육지식물보다 뛰어나다. 단위면적당 성장률도 높아 차세대 바이오메스로 큰 기대를 하고 있다.

진도에서 분리한 *Schizochytrium* sp. 1과 *Schizochytrium* sp. 2의 최적 성장 조건 확립을 위해 미세조류 성장에 있어서 필수요소인 영양원 농도와 light:dark cylce 로 인한 성장률을 관측하였다. 그 결과 *Schizochytrium* sp. 1의 경우 0.9 g/L에서 1.7 g/L로, *Schizochytrium* sp. 2는 0.39 g/L에서 0.8 g/L로 바이오매스가 증가됨을 알 수 있었고, 그에 따라 오일 축적능 향상을 위해 salt stress 조건의 조성하여 오일 축정능을 향상시켜 *Schizochytrium* sp. 1의 경우 약 21%, *Schizochytrium* sp. 1의 경우 약 42 %로 향상됨을 확인 할 수 있었다.

이와 함께 기존에 알려져 있는 미세조류 4종인 *Isochrysis garbana*, *Dunaliela*

salina, *Dunaliela tartiorector*, *Nannochloropsis oculata* 역시 성장률 증가를 위해 영양원인 nitrate 농도에 변화를 주어 바이오매스를 증가시켰으며, salt stress 환경을 조성하여 *I. garbana*의 경우 약 57%, *D. salina*의 경우 약 47%, *D. tartiorector*의 경우 약 54%, *N. oculata*의 경우 약 24%로 오일 축적능이 향상됨을 알 수 있었다.

또한, 어류 병원균의 비 유전성을 가지는 항생제 내성균인 persister cell에 관한 연구는 Persister cell은 기존의 항생제를 분해하는 저항균체(resistant cell)와는 다른 특성으로 항생제에 대한 저항성을 가지는데, 항생제가 존재하는 환경에서 새로운 기작으로 항생제에 대한 내성을 형성한다. 그래서 기존의 양식장에서 어류를 키울 때 어류에 투여하는 항생제는 일반적인 균주의 사멸 항생제 농도보다 더 높은 농도의 항생제를 투여하게 된다. 특히, *E. tarda*에 대한 다양한 항생제가 개발되어 있지만 내성균의 출현으로 그 효과가 좋지 않으며, 또한 persister cell에 의한 질병 재발을 방지하기 위해 균사멸 농도보다 훨씬 높은 농도의 항생제를 처리하고 있다.

Persiser cell의 특이적인 저감 효과를 확인 하기 위하여 선별된 3종의 식물 추출물(돌외, 예덕나무, 상산)을 항생제와 함께 사용하였으며, 돌외와 예덕나무가 100 μg/ml, 상산은 200 μg/ml의 농도에서 persister cell의 사멸 효과를 나타내었다. 또한 넙치를 이용한 12일간의 누적 폐사율을 조사한 결과, 식물 추출물과 항생제 혼합액의 복강 투여구가 항생제 단독의 복강 투여구 보다 낮은 누적 폐사율이 관찰되었고, 항생제에 첨가한 식물 추출물의 농도가 돌외 30 μg/ml, 예덕나무 10 μg/ml, 상산 10 μg/ml의 농도 투여구에서 가장 낮은 누적 폐사율을 나타내었다. 따라서 본 연구에 사용된 세가지 추출물들(돌외, 예덕나무, 상산)은 항균 활성을 가지지 않은 농도에서 항생제와 병용하여 persister cell의 저감 효과가 있음을 확인하였다.

CHAPTER I

Oil production from four microalgae

by changing culture conditions

Ⅰ. INTRODUCTION

Many studies have been carried out on biodiesel as a good replacement for fossil fuel. The advantages of biodiesel are a non-toxic, biodegradable and renewable fuel. Recently, some countries have enforced biodiesel blending to diesel fuel, such as BD5 and BD10 using 5 and 10% of biodiesel blending, respectively.

Currently, soybean oil and palm oil have been used as lipid to make biodiesel. Therefore, the necessity to develop nonedible biomass is increased. Thus, there is a rising interest on the production of biodiesel from marine microalgae. Microalgae can be grown fast using photosynthesis with carbon dioxide and the average content of oil per unit area is 10 times higher than terrestrial plants. Microalgae, as plants, store energy as carbohydrates and oil, and this oil is similar to those produced by crops such as soybean.

To produce high quantity of oil content, biomass production from microalgae should be enhanced by the optimization of light:dark cycle, light intensity, nitrate concentration and aeration rate (Jacob-Lopes et al., 2009; Hsieh et al., 2009). Oil accumulation in the cell body of microalgae occurs when microalgae are placed in environmental stress such as nitrogen deficiency (Hsieh et al., 2009). Several studies have been focused on the growth and oil accumulation of microalgae through cultivation in photobioreactors (Hsieh and Wu, 2009; Fernández et al., 2003). Oil is generally consisted of saturated and unsaturated fatty acids. Palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and

linolenic acid (C18:3) are known as common fatty acids for biodiesel production, especially, those fatty acids are strong candidates for improving the quality of biodiesel (Gerhard et al, 2008). However, few studies on fatty acid composition of the oil from microalgae have been carried out for biodiesel production (Lin et al., 2007).

The aim of this study is to evaluate cell growth, oil content and fatty acid composition of oil from six microalgae species, *Schizochytrium* sp. 1, *Schizochytrium* sp. 2, *D. salina, D. tartiorector, I. galbana* and *N. oculata* for biodiesel production in 2 L batch culture. Four species of microalgae have been used as live feeds for larvae culture in aquaculture (Lin et al., 2007) with stable cultivation and high nutritional value. The growth conditions on various nitrate concentrations were optimized to increase biomass production. After the optimization of culture conditions, 2 stage culture with various salt concentrations was carried out for the increase of oil content.

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Ⅱ. MATERIALS AND METHODS

1. Strain and culture condition

The microalgae samples were collected from the abalone-aquaculture of Jindo, Korea. The samples were cultured in modified F/2 medium and subjected to purification by serial dilution. Pure culture was obtained from single cell and the culture was established in F/2 medium, incubated at 20 ± 1°C under 21:3 light:dark cycle. The purity of the culture was ensured by repeated plating and by regular observation under microscope.

Four species of microalgae, *D. salina*, *D. tartiorector*, *I. galbana* and *N. oculata* were obtained from NLP Corp. (Busan, Korea).

2. Culture condition

The microalga used in this study was *Schizochitrium* sp. 1 and *Schizochitrium* sp. 2 isolated from Jindo, Korea. For the optimization of culture conditions of *Schizochitrium* sp. 1 and *Schizochitrium* sp. 2, cell growth was evaluated at different light intensities and nitrate concentrations at 20±1°C, 21:3 light:dark cycle and aeration in photo-bioreactors with a working volume of 2 L using modified f/2 media as described in Guillard and Ryther (1962). Nitrate concentrations and light:dark cycle were varied as N-free, 6.18, 12.37, 18.55 and 24.7 mg/L, 24:0, 21:3, 16:8, 12:12, 8:16, 3:21 and 0:24 hr, respectively. Four

species of microalgae, *D. salina*, *D. tartiorector*, *I. galbana* and *N. oculata* were obtained from NLP Corp. (Busan, Korea). All microalgae were cultured in the f/2 media (Guillard and Ryther, 1962), at 20 ± 1°C under 12:12 light:dark cycle as basic culture condition. Nitrate concentrations were varied as N-free, 6.18, 12.37, 18.55 and 24.7 mg/L.

3. Analysis method

3.1 Measurement of biomass for cell growth

For dry cell weight determination, 1 ml of culture samples in triplicate were filtered through pre-weighted 1.2 μm glassfiber filters (Whatman GF/C; 47mm) to remove water. The retained cells were washed twice with distilled water and dried using a freeze dryer to constant weight.

3.2 Measurement of nitrate

Nitrate concentration was determined using UV-Vis spectrophotometer (Ultrospec 6300 pro, Amersham Biosciences, Sweden) at OD220 (Collos et al., 1999). The samples were centrifuged at 9809×g, 4°C for 10 min. The supernatant was used for the measurement of nitrate concentration. A standard curve was constructed and used for the determination of nitrate

concentration.

3.3 Harvest of biomass

The microalgae were then centrifuged at 8000 rpm, 4℃ for 10 minutes. The pellet was washed using distilled water, and then dried using a freeze dryer for 48 hr. Dry cell weight was measured and grounded using a mortar. The pellet was weighed and considered as dry cell weight (DCW).

3.4 Lipid extraction

Total lipids were extracted using chloroform: methanol (2:1, v/v) as extraction solvent by shaking incubator. The organic solvent was evaporated, and extracted lipids were dried at 80℃ to constant weight. Extracted oils from the culture sample were properly dried until reaching constant weight. Total lipids content was obtained as % of dry cell weight.

$$\text{Lipid content (\% dcw)} = \frac{W\ (g) - W_0\ (g)}{DCW\ (g)} \times 100$$

3.5 Fatty acid analysis

To analyze fatty acids, extracted crude lipid was methylated. The extracted lipid was weighed and 1% sulfuric-methanol was added to extracted lipid. Extracted oil and 1% sulfuric-methanol mixture were heated

at 50 ℃ for overnignt for transesterification and cooled at room temperature in desiccator. Fatty acid methyl ester was extracted using of hexane. The methyl ester was analyzed by the modified method of Liu et al. (2001) via gas chromatography (Thermo Finnigan Trace GC, San Jose, CA, USA) equipped with a flame ionization detector (FID) using silica capillary column (007-CW-30-0.25F). The initial temperature was 100°C, followed by a program that increases temperature 5°C min-1 to a final temperature of 200°C. The injector and detector temperature were 200°C and 250°C, respectively. N_2 was used as low gas, and gas pressure was 65 psi. The quantity of fatty acid was calculated by peak areas with standard curves of palmitic acid, stearic acid, oleic acid, linoeic acid and linolenic acid which were obtained from Sigma-Aldrich (St. Louis, MO, USA).

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Ⅲ. RESULT AND DISCUSSION

1. Cell growth and lipid content of *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2

The experiment was performed in 3 L photo-bioreactors with 2 L of f/2 medium. The time course of biomass and lipid content by *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 is shown in Fig. 1. *Schizochytrium* sp. 1 produced biomass 0.9 g/L and *Schizochytrium* sp. 2 produced biomass 0.39 g/L at 20 days. *Schizochytrium* sp. 1 was produced 15% of lipid content and *Schizochytrium* sp. 2 was produced 23% of lipid content at 20 days. The fatty-acid profiles are shown in table 1. Palmitic acid production had 264.4mg/g 283.1 mg/g of biomass on day 20 with *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2. A low amount of other fatty acids was produced by *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2.

Schizochytrium sp. 1 and *Schizochytrium* sp. 2 is a spherical single-cell organism and growth and metabolites production are influenced by cultivation conditions. The Palmitic acid yield and the predominant fatty acid produced was about 264.4mg/g 283.1 mg/g, respectively. There were lower yields of the various C18 fatty acids by *Schizochytrium* sp. and *Schizochytrium* sp. 2. The same strain was studied by Singh et al. and had a lower Palmitic acid yield (<100 mg/L) under pH 7 and 25℃ after 5 days orbital shaking at 200 rpm (Singh A et al. 1996). The different results shown that varying the cultivation conditions profoundly affect cell growth and fatty-acid

production by these microorganisms.

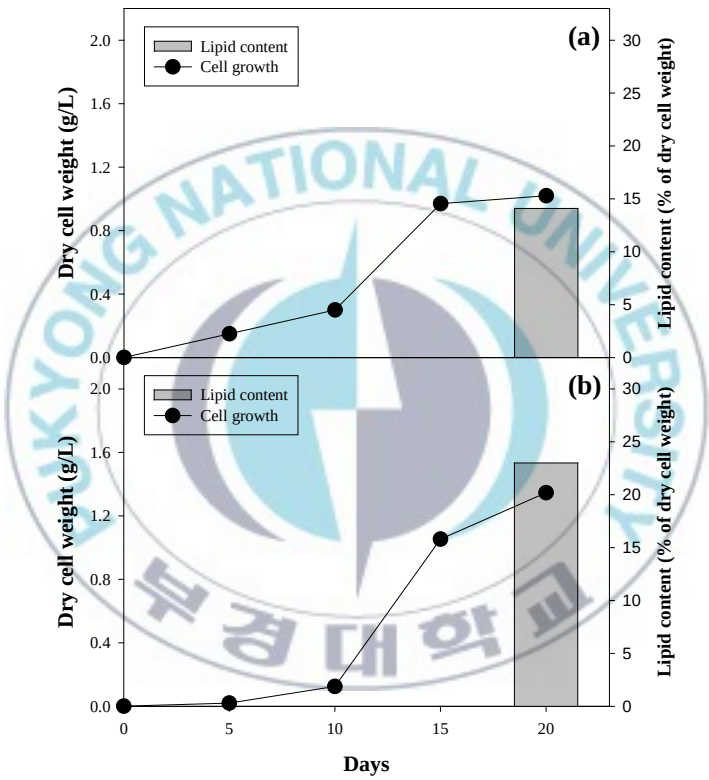

Fig. 1. Result of biomass and lipid content of (a) *Schizochytrium* sp. 1 and (b) *Schizochytrium* sp. 2.

Table 1 Fatty acid compositions of *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 on basic culture condition

Cell / Fatty acid	*Schizochytrium* sp. 1	*Schizochytrium* sp. 2
C14:0	14.6±0.3	15.5±0.2
C16:0	264.4±0.3	283.1±0.5
C18:0	11.1±0.0	11.0±0.4
C18:1	2.8±0.4	3.1±0.3
C18:2	1.7±0.0	2.3±0.2
C18:3	22.0±0.1	33.3±0.3
C20:0	1.1±0.1	1.1±0.1
C22:0	1.1±0.0	1.0±0.1
Total fatty acid	318.8±1.2	350.4±1.7

2. Optimization of growth condition

2.1 Effect of nitrate concentrations

To optimize nitrate concentration for the growth, various nitrate concentrations of N-free, 6.18, 12.37, 18.55 and 24.74 mg/L were applied to the culture as shown in fig. 2. *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 with various nitrate concentrations of 12.37, 18.55 and 24.74 mg/L and reached to the stationary phase on 12th day as showed in Fig. 2. Nitrate was consumed early phase of the culture by *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 at the condition with low nitrate concentrations as shown in Fig. 2. Nitrate concentration of 24.74 mg/L did commonly produce higher biomass comparing to other nitrate concentrations. *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 showed the maximum biomass production of 1.7 g/L and 0.8 g/L with 24.74 mg/L. *Schizochytrium* sp. 1 showed the biomass production of 0.934g/L with N-free. It would be due to stored the nitrate in the cell body during *Schizochytrium* sp. 1 was cultured in pre-cultivation. The results imply that various nitrate concentrations influence culture period, biomass production.

Therefore, nitrate concentration of 24.74 mg/L was applied to the culture for high biomass production in further study.

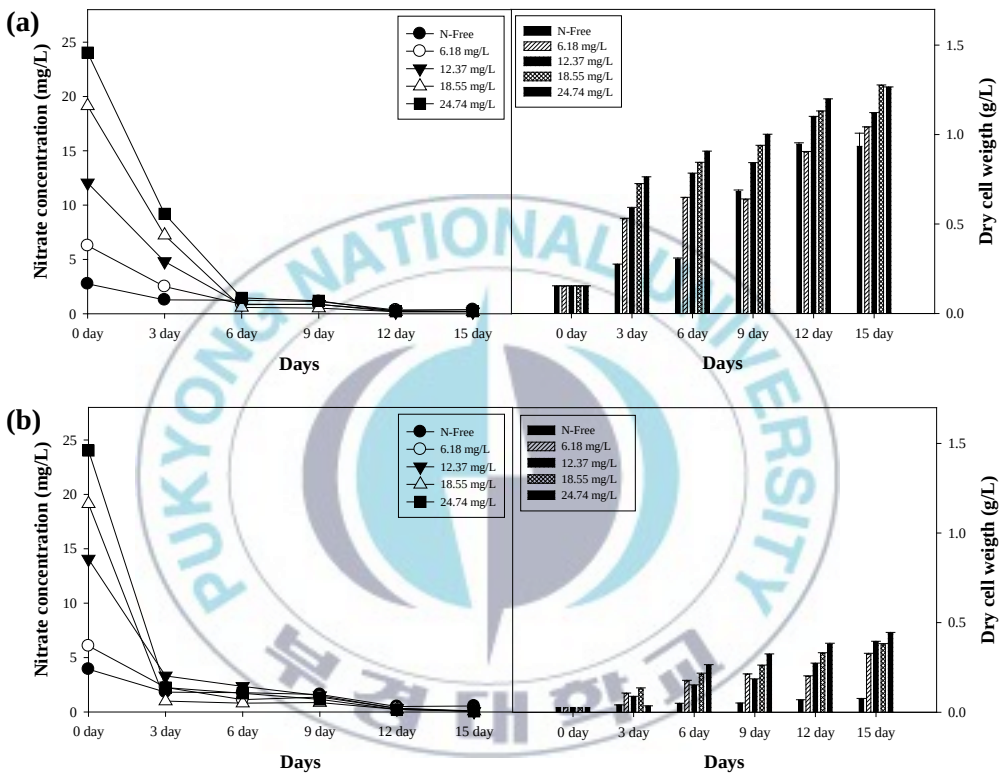

Fig. 2. Different of growth and nitrate concentration from (a) *Schizochytrium* sp. 1 and (b) *Schizochytrium* sp. 2 on various nitrate concentrations

2.2 Effect of light and dark cycle

Schizochytrium sp. 1 and *Schizochytrium* sp. 2 was cultured on various light:dark cycles to increase biomass production and reduce culture period as shown in Fig.. 4. *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 with 24:0 light:dark cycle was faster to reach the stationary phase on 6th day. Moreover, nitrate was almost absorbed by *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 with 24:0 light:dark cycle in 12 days from the inoculation. Nitrate was almost consumed by *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 on various light:dark cycles except for the condition of 4:20 and 0:24 light:dark cycle which remained the nitrate concentration (data not shown). *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 could consume nitrate faster and showed high biomass production at the conditions of high light:dark cycles.

The tendency of biomass production of 0.78 g/L on various light:dark cycles from the present study showed similar trends to those of previous study (Jacob-Lopes et al., 2009). It indicates that the biomass production could be increased by high light:dark cycles.

Therefore, the condition of 24:0 light:dark cycle was used for the further study to increase biomass production.

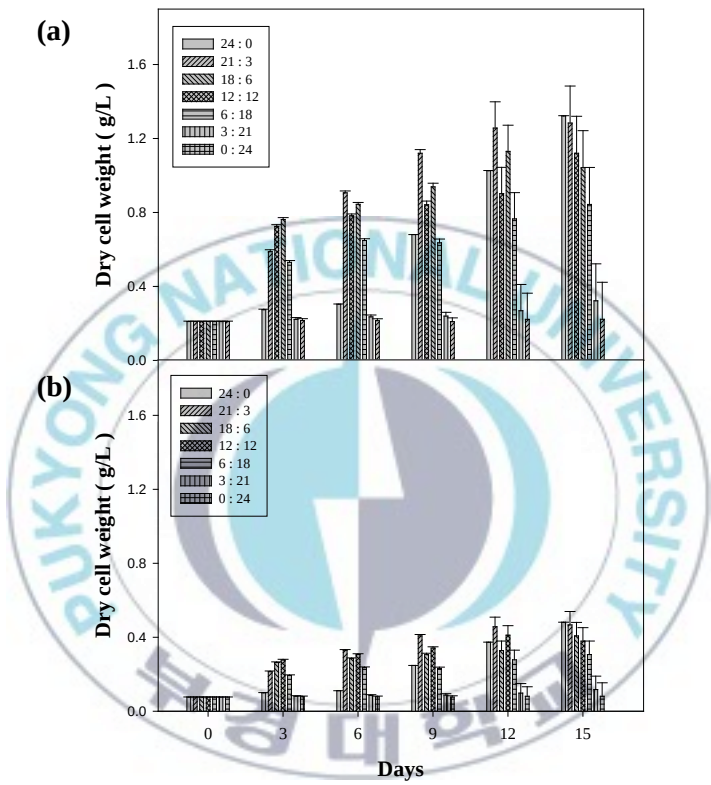

Fig. 3. Different of growth from *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 on various light:dark cycle

3. Two-stage of microalgae

Under unfavorable environmental or stress conditions, microalgae shift their lipid biosynthetic pathways towards the formation and accumulation of neutral lipids, mainly in the form of TAGs (Hu et al., 2008). Unlike the glycolipids and phospholipids found in membranes, TAGs do not perform structural role but instead serve primarily as a storage form of carbon and energy (Pruvost et al., 2009). After synthesis, TAGs are deposited in densely packed lipid vacuoles located in the cytoplasm of the microalgae as shown in Fig. 4. In this study, second stage process for TAG accumulation was carried out with the culturing benthic microalgae at salinity range from 0 to 50 ‰ and optimal culture conditons. All groups were depleted nitrogen in the culture. Oil content of microalgae was 15% and 24 % with *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 before the second stage with stresses. The condition of 20 ‰ with optimal culture conditions showed high oil accumulations of 21% at 4 days with *Schizochytrium* sp. 1 as shown in Fig. 8 (a). However, the condition of 0 ‰ with optimal culture conditions showed high oil accumulations of 42% at 2 days with *Schizochytrium* sp. 2 as shown in Fig. 8 (b).

However, at 4 days, the biomass was decreased by the cell degradation, therefore, oil contents did not increased significantly at the salinity of 40 ‰ and 50 ‰ as shown in Fig. 8. Microalgae usually suffer rapid degradation of the photosynthetic membrane with accumulation of cytosolic TAGs-enriched lipid bodies in stress conditions. In the second stage process, light energy would not use the energy source for the photosynthesis, but stimulated TAGs accumulation of the microalgae

as a carbon source. The condition of nitrogen depletion is known to effect for the accumulation of lipid and TAG content in microalgae (Hu et al., 2008; Go et al., 2011). Coombs et al. (1967) reported that the lipid content of the diatom *Navicula pelliculosa* increased by about 60% during a 14 h silicon starvation period.

Therefore, this result showed that oil content of the *Schizochytrium* sp. 1 increased from 15 % to 21 % with the conditions of nitrogen depletions and salinity stress of 20 ‰ during 4 days without loss of biomass and *Schizochytrium* sp. 2 increased from 24 % to 42 % with the conditions of nitrogen depletions and salinity stress of 0 ‰ during 2 days without loss of biomass.

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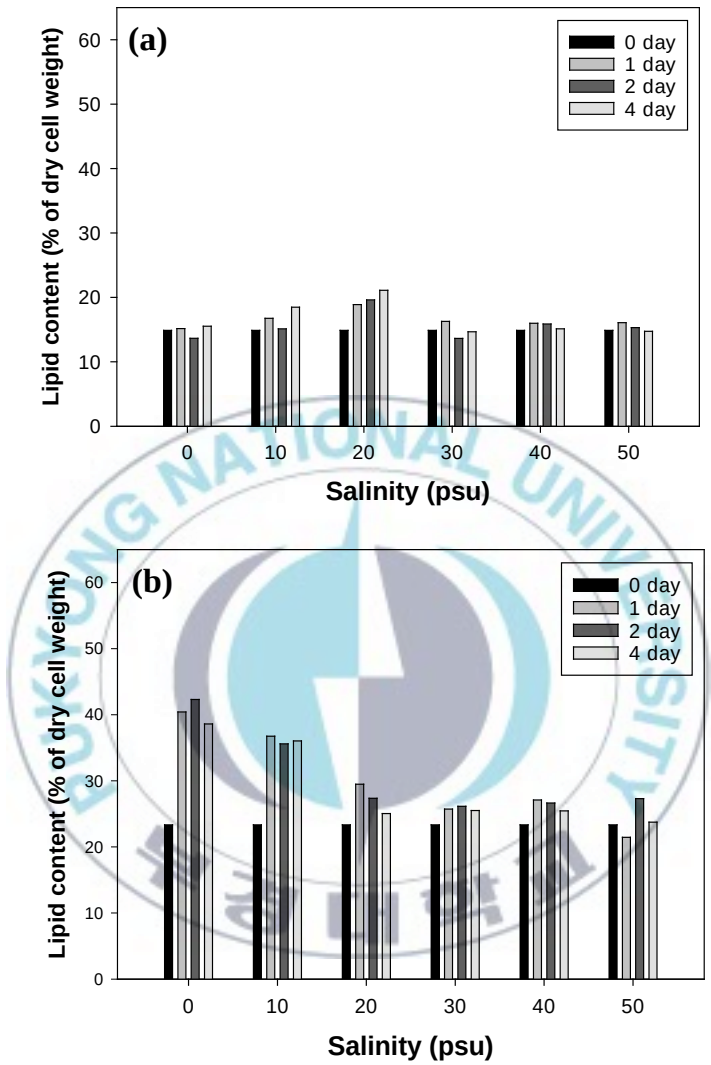

Fig. 4. Result of lipid content on various salinity with (a) *Schizochytrium* sp. 1 and (b) *Schizochytrium* sp. 2

4. Growth and lipid content of four microalgae

Fig. 1 showes cell growth curves of four microalgae, *N. oculata*, *I. galbana*, *D. tertiolecta* and *D. salina* with basic culture condition. The biomasses of 0.62 g /L and 0.49 g dcw /L were observed in *I. galbana* and *N. oculata* on 11 days after the inoculation as shown in Fig. 1. The biomasses of 0.23 g dcw /L and 0.21 g dcw /L were observed in *D. tertiolecta* and *D. salina* on 14 and 11 days after the inoculation as shown in Fig. 7.

The oil contents for four marine microalgae at 19 days of culture after the stationary phase were shown in Fig. 8. The oil content of 23.15% of dry cell weight from *I. galbana* was obtained using f/2 medium with NaNO3 as N source. *I. galbana* showed that oil content reached to 24.65% of dry cell weight with modified Walne medium containing NH4NO3 as N source (Lin et al., 2007). The oil content in this study with *I. galbana* was consistent to the study of Lin et al. (2007). It suggested that NaNO3 in f/2 medium and NH4NO3 in Walne medium produced similar oil content *to I. galbana*. *N. oculata* showed the oil contents of 8.23% of dry cell weight on 19 days after the stationary phase. 2. *D. tertiolecta* and *D. salina* showed the oil contents of 18.04% and 16.32% of dry cell weight on 19 days after the stationary phase. Eight fatty acids composition and oil content after the stationary phases of four microalgae cultures were shown in Table 1.

As shown in Table 1, high palmitic acid content of 364.03, 133.1, 104.6 and 146.07 mg /g oil was observed in *I. galbana*, *D. tartiorecta*, *N.ouclatla* and *D.salina*

from stationary phase of the cultures. High linolenic acid content of 232.69, 98.6, 123.47 and 113.69 mg /goil was also observed in all four microalgae from stationary phase as shown in Table 1. Mistric acid, stearic acid, oleic acid, linoleic acid, arachidic acid and beneic acid contents were lower than 50 mg /goil in the four microalgae as shown in Table 1. Palmitic acid content of N.ouclatla was to 104.60 mg /goil from stationary phase. However, oil content of N.ouclatla remained at low level of 8.23% of dry cell weight. The fatty acid composition of microalgae could be easily changed by controlling the culture condition (Lin et al., 2007; Toron et al., 2002). The palmitic acid and stearic acid contents of Chlorella sp. was changed significantly by the culture conditions during the 5 days of cultivation period (Johnson et al., 2009). It suggests that changes on specific fatty acid contents in total fatty acids are dependent on species characteristics, culture condition and period. The palmitic acid, stearic acid and particularly oleic acid have been recognized as the most common components of biodiesel (Gerhard, 2008). Palmitic acid contents were increased and unsaturated fatty acids were decreased during post-exponential phase to stationary phase in *Schizochytrium limacinum* SR21 and Nannochloropsis oculata cultivation (Morita et al., 2006; Hodgson et al., 1991). Four microalgae in this study showed the high palmitic acid content in stationary phase. Microalgae uses unsaturated fatty acids as energy sources and accumulates saturated fatty acids when nutrients deficiency occurs in the culture medium (Jeh et al., 2007).

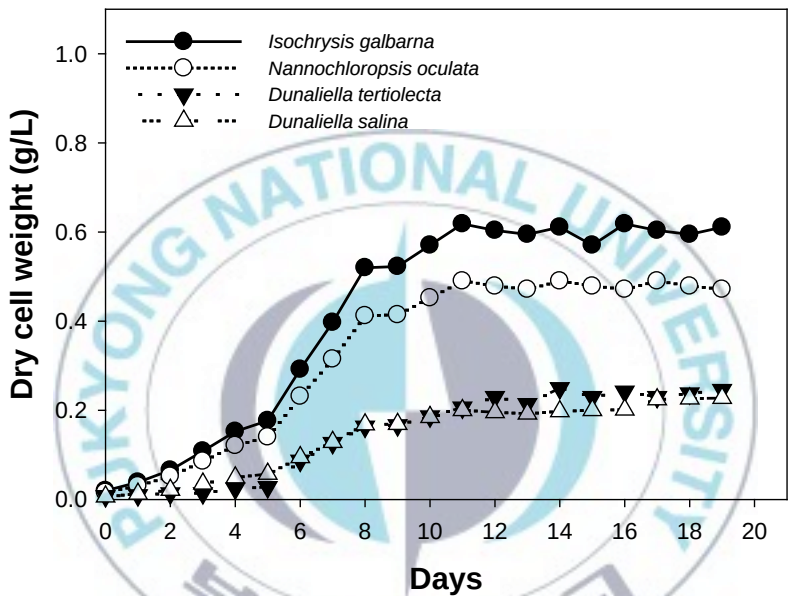

Fig. 5. Growth curve of *N. oculata*, *I. galbana*, *D. tertiolecta* and *D. salina* in basic culture condition

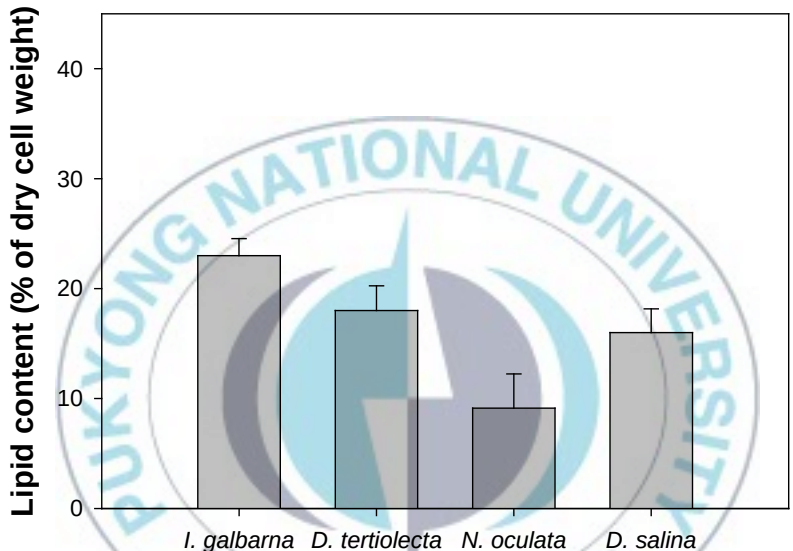

Fig. 6. Result of lipid content from microalgae, *I. galbana, D. tertiolecta, N. oculata* and *D. salina* in basic condition

Table 2 Fatty acid compositions (mg/g oil) of *I. galbana*, *D. tertiolecta*, *N. oculata* and *D. salina* with basic culture condition at 20℃ under light:dark cycle of 12:12 for 19 days

Fatty acid \ Cell	*I. galbana*	*D. tertiolecta*	*N. oculata*	*D. salina*
C14:0	4.6±0.3	1.1±0.1	5.7±0.7	4.6±0.3
C16:0	364.4±0.3	133.1±1	104.60±0.3	146.07±0.1
C18:0	44.78±0.7	6.7± 0.3	7.2±0.3	44.78±0.8
C18:1	35.12±0.8	3.9± 1.0	11.35±0.5	26.91±0.7
C18:2	20.84±0.4	9.8± 1.4	46.87±0.8	10.23±0.2
C18:3	232.69±0.1	98.6 ±2.3	123.47±0.9	113.69±0.7
C20:0	2.4±0.1	3.1±0.1	1.3±0.4	2.7±0.1
C22:0	9.6±0.5	1.8±0.5	9.3±0.8	19.6±0.5
Total fatty acid	714.43±3.1	258.1±6.8	309.79±4.7	368.58±1.9

5. Effect of nitrate concentration on cell growth

To optimize nitrate concentration for the growth, nitrate concentrations of N-free, 6.18, 12.37, 18.55 and 24.74 mg /L were applied to the culture as shown in Fig. 3. *I. galbana* with nitrate concentrations of 12.37, 18.55 and 24.74 mg /L reached to the stationary phase on 12 days as shown in Fig. 3(a). *N. oculata* with 12.37, 18.55 and 24.74 mg/L reached to the stationary phase on 10, 16 and 14 days as shown in Fig. 3(c). *D. salina* with nitrate concentrations of 12.37, 18.55 and 24.74 mg /L reached to the stationary phase on 10 days and 6.18 mg /L nitrate reached to the stationary phase on 14 days as shown in Fig. 3(b). *D. tartiorector* with nitrate concentrations of 6.18 and 12.37 mg /L reached to the stationary phase on 12 and 14 days and 18.55 and 24.74 mg /L nitrate reached to the stationary phase on 10 days as shown in Fig. 3(d). *I. galbana* culture with nitrate concentration of 24.74 mg /L produced biomass 0.82 g dcw /L. *N. oculata* with 24.74 mg /L nitrate produced biomass 0.51 g dcw /L. *D. salina* culture with nitrate concentration of 18.55 mg /L produced biomass 0.29 g dcw /L. *D. tertiolecta* produced biomass of 0.31 g dcw /L with 24.74 mg /L nitrate on stationary phase. In this result, *D. salina* and *D. tertiolecta* produced low biomass in comparison to biomass production of *I. galbana* and *N. oculata*.

The results imply that various nitrate concentrations influenced culture period, biomass production and oil production. Therefore, *I. galbana*, *N. oculata* and *D.*

tertiolecta showed high biomass with 24.74 mg /L of nitrate concentration. This result was applied to the culture for high biomass and oil production in further

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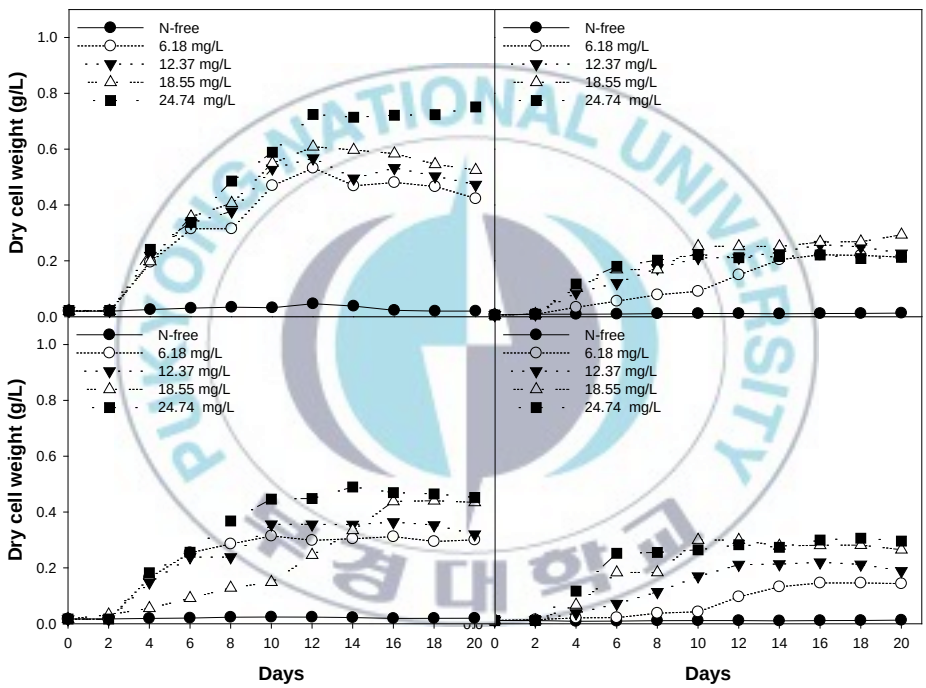

Fig. 7. Changes of nitrate concentrations on dry cell weight with (a) *I. galbana*, (b) *D. salina*, (c) *N. oculata* and (d) *D. tertiolecta*

6. Two stage cultivation

Fig. 4 was oil production from four microalgae with salt stress of 2nd stage culture after the stationary phase. The various salt concentration of culture for oil accumulation was carried out with the culturing four microalgae at salinity range from 0 psu to 50 psu. Second stage culture was carried after stationary phase, the initial oil contents of the microalgae were 23.15% oil of *I. galbana* (g×100 /g dcw), 16.32% of *D. salina*, 8.23% of *N. oculata* and 18.04% of *D. tariolecta* before the 2nd stage with optimal nitrate concentration. *I. galbana* showed high oil content of 57 % at 2 days with 0 psu. *D. salina* showed 47% at 2 days with 0 psu. *N. oculata* and *D. tariolecta* showed oil content of 28 % and 55 % at 4 days and 2 days with 0 psu, respectively.

Therefore, total oil contents could increase at the salinity of 0 and 10 psu as shown in Fig. 4. Microalgae usually suffer rapid degradation of the cell membrane with the accumulation of cytosolic oil bodies in stress conditions. In the 2nd stage of culture, light energy would not be used to the energy source for the photosynthesis, but stimulated oil accumulation of the microalgae as a storage carbon source. The condition of nitrogen depletion has been used for the accumulation of oil content in microalgae (Lee et al., 2011; Go et al., 2011; Hu et al., 2008). Coombs et al. (1967) reported that the oil content of the diatom *Navicula pelliculosa* increased by 60% during 14 hour salt starvation. Takagi et al. (2006) reported that the oil content of microalgae was changed in response to

environmental salinity. Therefore, this result showed that oil contents of the four microalgae, *I. galbana, D. salina, N. oculata* and *D. tariolecta* increased from 23% to 57%, from 18% to 47%, from 15% to 38% and from 15% to 55% with the conditions of salt stress conditions 0 and 10 psu during 0-2 days. From this study, *I. galbana* with high oil content were chosen for the production of oil.

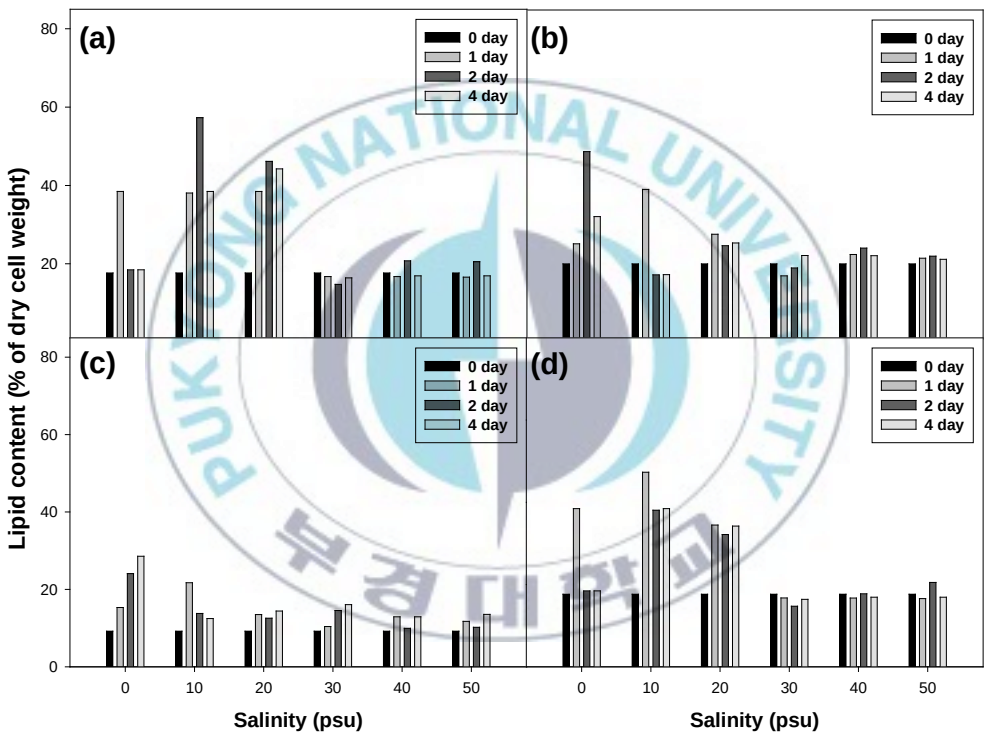

Fig. 8. Results of salt stress culture for lipid accumulation on (a) *I. galbana*, (b) *D. tertiolecta*, (c) *N. oculata* and (d) *D. salina* during 4 days

Ⅳ. CONCLUSION

For biomass production and oil accumulation, nitrate concentration and light : dark cycle for *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 culture were optimized at 24.75 mg/L and 24:0, respectively. The optimal conditions led to a biomass production of 1.7 g/L and 0.8 g/L with *Schizochytrium* sp. 1 and *Schizochytrium* sp. 2 in 12 days to reach the stationary phase in a 2 L batch culture. *Schizochytrium* sp. 1 showed oil content of 21% on 20 psu was obtained at 2nd stage culture. *Schizochytrium* sp. 2 showed oil content of 42% on 20 psu was obtained at 2nd stage culture.

I. galbana showed high cell growth comparing to *D. tartiolecta, D. salina* and *N. oculata*. *D. tartiolecta* showed high biomass with 24.74 mg /L of nitrate concentration and oil content of 54% on 0 psu was obtained at 2nd stage culture. *D. salina* showed high biomass with 18.55 mg /L of nitrate concentration and oil content of 47% on 10 psu was obtained at 2nd stage culture. *I. galbana* showed high biomass with 24.74 mg /L of high nitrate concentration and oil content of 57% on 10 psu was obtained at 2nd stage culture. *N. oculata* showed was high biomass with 24.74 mg /L of nitrate high concentration and oil content of 38% on 10 psu was obtained at 2nd stage culture.

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CHAPTER II

Effects of natural compounds from various plants eradicate the persister cell of *Edwardsiella tarda* treated with antibiotics of florfenicol and amoxicillin

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Ⅰ. INTRODUCTION

Recent antibiotic misuse is a major problem worldwide and the FDA and WHO has recommended a reduction in the use of antibiotics for food crops, livestock and fish. Persister cell was first reported in 1944 by Joseph Bigger (Bigger, J. W., 1944) and *Staphylococcus aureus* resistant have different mutations (resisterant) resistance mechanisms to penicillin and was named persister cell (Bigger, J. W., 1944). Persister cell was different from other characteristics to resistant cell decomposition to conventional antibiotics, but antibiotics existed environment which new molecular mechanism of persister cell not apoptosis by forming a resistance to antibiotics. TisB expressed from Toxin-Antitoxic module(TA module) is formed by substance to antibiotics or induced DNA damage which reduces the ATP level reduced Proton Motive Force as persister cell metabolic range for minimizing the dormant-forming environment.

Persister cell is induced dormant state at expressed gene in environment that exists antibiotics.If the presence of antibiotics are dormant persister cell division and metabolic activity gets active again disappears antibiotics (Lewis, K., 2007; Maisonneuvea, E. et al., 2011) . Therefore, the cause of disease recurrence, persister cell is being given large amounts of antibiotics to try to remove it, have been the cause. For Persister cell formation mechanism is still exactly unknown but, on these persister cell research has been a lot of research being carried out in order to reduce the use of antibiotics.

Pathogenic strains used in this study as *E. tarda* in freshwater and seawater fish to Edwardsiellosis induces. *E. tarda* increase of the onset of symptoms to the symptoms, such as abdominal swelling, hernias, and flounder in the form of inflicting the most damage as pathogenic bacteria, mainly adhere to the warmth that appears in many diseases (Kim, J. S., et al., 2001: Kusuda, R., et al., 1998). *E. tarda* in a variety of antibiotics have been developed, but its effect is not desirable, as the emergence of resistant strains, bacteria sterilizing concentration much higher than the concentration of antibiotics in order to prevent a recurrence of the disease due to persister cell processing and (Heo, J. H., et al., 2002)

Persister cell of antibiotic resistance reduction effect experiment using 10 kinds of plant extracts in this laboratory. Persister cell representing the reduction effect of 10 kinds of plant extracts, extract of *Gynostemma pentaphyllum* (돌외), *Mallotus japonicas* (예덕나무), *Orixa japonica* (상산) could be confirmed. Others are perennial plants, stones saponins (사포닌) series compounds gypenosides, the direction of ingredients and phyllodulcin flavonoids, carotene, containing (Kuwahara, M., et al., 1989; Shah, D., et al., 2006). Also *M. japonicas* in leaf, deciduous small arboreal containing rutin, isoprenoid derivatives and geranin, mallotusinic acid, mallotinic acid (Lim, H. K., et al, 1999; Saijo, G., et al., 1990), *O. japonica* are not deciduous shrubs belonging to Rae known components. Persister cell reduction effects, but most of the plant extracts used alone, when used in conjunction with antibiotics persister cell reduction effects are reported increase. Persister cell death

and high reduction effect by using a combination of antibiotics and plant extracts extracts Therefore, in this study is to find, *in vivo* experiments using in vitro and flounder through a reduction in persister cell efficacy was confirmed.

Ⅱ. MATERIALS AND METHODS

1. Basic culture conditions

Used in this study *Edwardsiella tarda* NH1 Ed ward increased mortality flounder isolates and separated from the other lab strains had been preserved from the sale were used under Tryptic Soy Broth (TSB, Difco, Detroit, USA) in medium containing 1.5% NaCl, the composition of the basic medium was used for 24 hours, 215 rpm, 30 ℃ were cultured under conditions (Choi, S, H. et al., 2011; Choi, S.H., et al., 2010).

2. Plants extract and composition of antibiotics

10 kinds of plant extracts, when they were treated using HTS (high throughput screening) system, such as antibiotics and plant extracts from Sungkyunkwan University Protein Engineering Laboratory, *E. coli* persister cell reducing effect of screening of the plant extract shown that the sale received 10 kinds of plant extracts used in the experiment shown in Table 1. Plant extracts in solvent DMSO (Dimethyl sulfoxide, Sigma) was used at a concentration of 1 mg / ml. In this experiment, the antibiotic used for the treatment of streptococcal syndromes, and Ed ward bottle of Fisheries for AF-(DaeSung Microbilogical Labs Co., LTD, Korea) was used, the amoxicillin 100 mg / ml and florfenicol 100 mg / ml are mixed.

3. Reduction effect of persister cell

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4. Experimental control and the rearing tank.

Experimental fish used in this study were purchased from the coastal fisheries in Yeosu, two weeks to be able to adapt to the experimental environment, the average water temperature from 20 ℃ to 19 ℃ and keeping domesticated were used in the experiments. Fry flounder (average initial weight: 25.6 ± 1 g) after the preliminary forty-six each 15 rats randomly selected a total of 20 square plastic tank experiments were carried out. Seawater filtered through 0.1 μm filters used in the experiment were fed

during the whole experiment period, the average water temperature at 21 ℃ in the range of 23 ℃ to maintain the proper water temperature.

5. *In vivo* test

Way to check administered antibiotic mixture of plant extracts Persister cell reducing effect in Juvenile flounder administered via intraperitoneal injection method. Prior to administration, E. tarda pathogen concentration of 4.0 × 104 CFU / ml, 0.1 ml / fish with the liquid each attack test was carried out. Days after each attack after the test concentrations of each antibiotic mixture of plant extracts and mixed injections. Flounder on the attack of the test results showed cumulative mortality 12 days.

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Ⅲ. RESULT AND DISCUSSION

1. Persister cell formation

Maintain the viability of the cells at a high concentration of antibiotics persister cell, and the cell number was maintained constant. Fig. 1 and *E. tarda* gradually decreased, but increased with concentration of antibiotics in the culture medium of CFU showed that more than 25 μg / ml of the antibiotic concentration is maintained constant CFU. This E. tarda pathogen that remains constant in the high antibiotic concentrations seen with increasing concentrations of antibiotics that persister cell interval to keep a constant, regardless of CFU formation is considered (Keren, I., et al., 2004).

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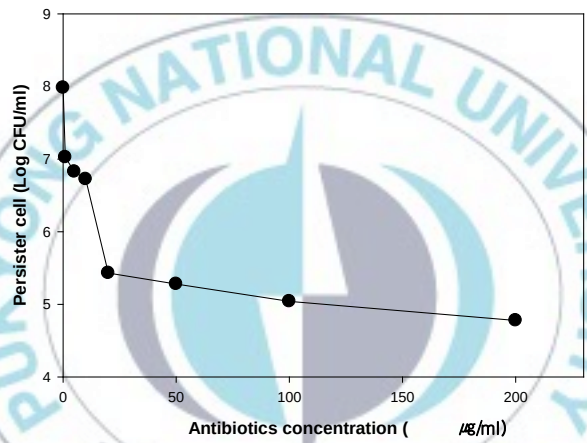

Fig. 1. Persister cell formation of *E. tarda* after the infection and the treatment of florfenicol and amonicillin in the ranges of 1 to 200 ug/ml incubated for 16 hours at 30 °C

2. Plant extracts by the addition of *E. tarda* growth curve

10 kinds of plant extracts (Table 1) were cultured alone added when E. tarda pathogen growth curves were compared. Of persister cell-specific effect in reducing Learn which plant extracts, when added to antibiotics, antibacterial plant extracts were screened. *G. pentaphyllu*, *M. japonicas* and *O. japonica* from fig. 2. shown, as there is no difference in the growth curve of the experimental growth curve and control of *E. tarda* addition of plant extracts alone showed no antibacterial plant extracts, which in itself shows that, because of these persister cell reduction was to determine the effect.

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Table 1 List of natural plant compound used in this study

Scientific name	Used part
Magnolia obovata	stem-bark
Alnus firma	flower
Panax ginseng	root
Gynostemma pentaphyllum	whole Plant
Mallotus japonicus	fruit
Cuscuta japonica	whole Plant
Sophora flavescens	aerial part
Pinus koraiensis	root
Magnolia obovata	leaf
Orixa japonica	leaf,stem,flower

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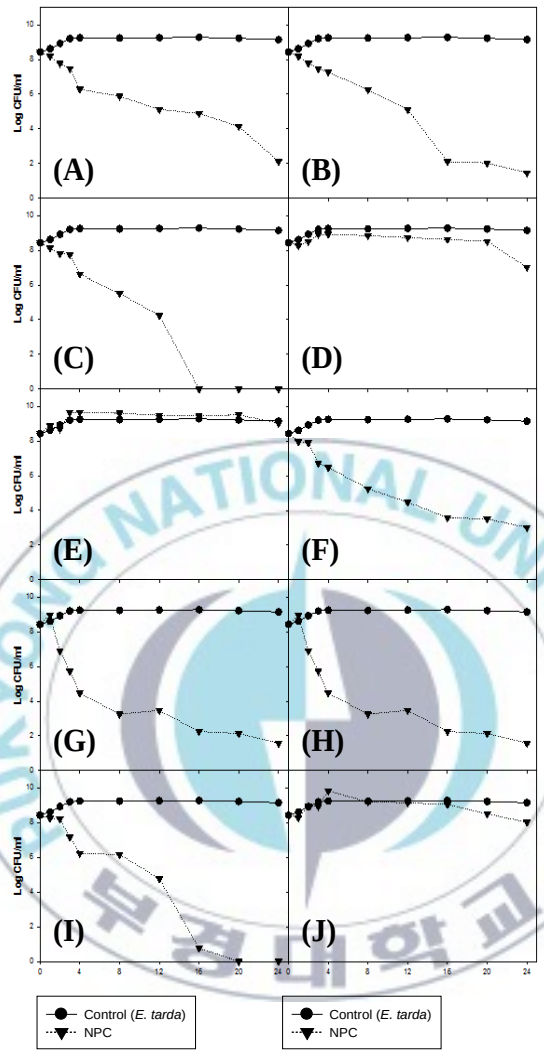

Fig. 2. Cell growth of *E. tarda* to select natural plant compounds without antibiotic (florfenicol, amonicillin) property cultured at 30 ℃ in shaking incubator (A) *M. obovata*, (B) *A. firma*, (C) *P. ginseng*, (D) *G. pentaphyllum*, (E) *M. japonicas*, (F) *M. japonicus*, (G) *S. flavescens*, (H) *P. koraiensis*, (I) *M.*

obovata, (J) *O. japonica*

3. Check the effect of the reduction of a mixture of antibiotics and plant extracts of persister cell.

Already known about the combination of antibiotics and plant extracts research persiser cell specific effect in reducing [8] In the present study, to confirm the most antibacterial plant extracts used to determine the effect of the reduction of pathogens, but Fig. 3. As shown in this picture, species of plant extracts antimicrobial activity screening was used in conjunction with antibiotics. As a result, *O. japonica* and *M. japonicas* showed effect of persister cell death at a concentration of 200 μg/ml and 100 μg/ml, respectively. Persister cell formed by antibiotics reducing effect, which added three species of plant extracts believes that it is improving.

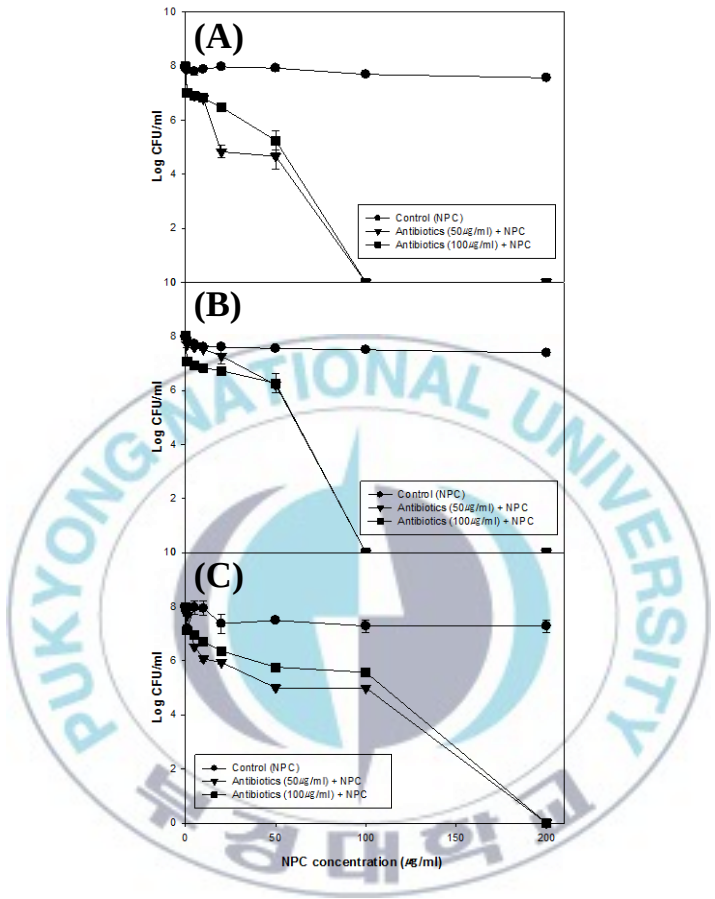

Fig. 3. Effect of natural plant compounds with antibiotics (florfenicol, amonicillin) to kill persister cells in shaking incubator for 16 hours at 30 °C (A) *G. pentaphyllum*, (B) *M. japonicas*, (C) *O. japonic*a; NPC = natural plant compound

4. Time dependent test

Reduction over a period of time of three species of selected antibiotics and plant extracts, as shown in Fig.4. Persister cell reducing effect of all the three species of plant extracts complete 16 hours of apoptotic happened was, the reduction effect, the difference was reduced from 4 to 12 hours.Ie, 8 hours for of *G. pentaphyllum M. japonicas* since the reduction of the effect of *O. japonica* was confirmed to appear after 2 hours from the reduction effect of persister cell.

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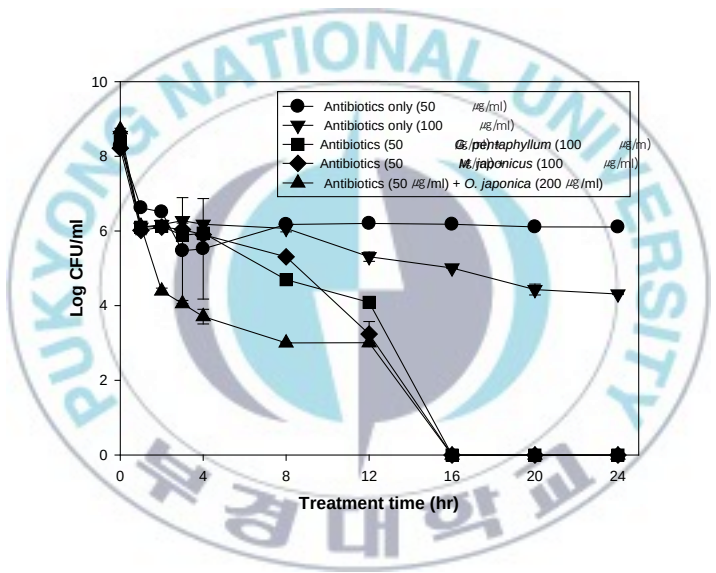

Fig. 4. Time dependent killing effect of *E. tarda* with the addition of natural plant compounds with antibiotics (florfenicol, amonicillin) incubated at 30 ℃ with shaking

5. Flounder by using *E tarda* persister cell for the reduction of *in vivo* test

In vitro test persister cell reduction effects through a combination of plant extracts and antibiotics identified three species of plant extracts using flounder by using a 12 days cumulative mortality was investigated. As a result, plant extracts and antibiotics intraperitoneal injection of a mixture of the antibiotics alone peritoneal antibiotics added to the administration group showed a lower cumulative mortality, *G. pentaphyllum* 30 μg/ml, the concentration of plant extracts *M. japonicas* 10 μg/ml, *O. japonica* 10 μg/ml concentration in the administered nine lowest cumulative mortality was observed Fig. 5. Also showed survival rates similar to the control at a concentration of plant extracts intraperitoneally administered plant extracts used in the present study, the concentration, the judge was not toxic to fish. It also adversely affects the activity between intraperitoneally administered extract with a concentration higher than the concentration used in the present study in the paper (Yook, C.S., et al., 1990). *G. pentaphyllum* case, but rather when administered in the serum increased antibody production in the spleen cells were a cytokine that is secreted was also increased. *M. japonicas*, acute toxicity has been reported (Roberts, M. E., et al., 2005) worry about the acute toxicity and chronic toxicity tests that do not represent the tests used in this study, unlike those known to be toxic seeds in the fruit middle leaves, roots, is considered to be non-toxic flower parts.

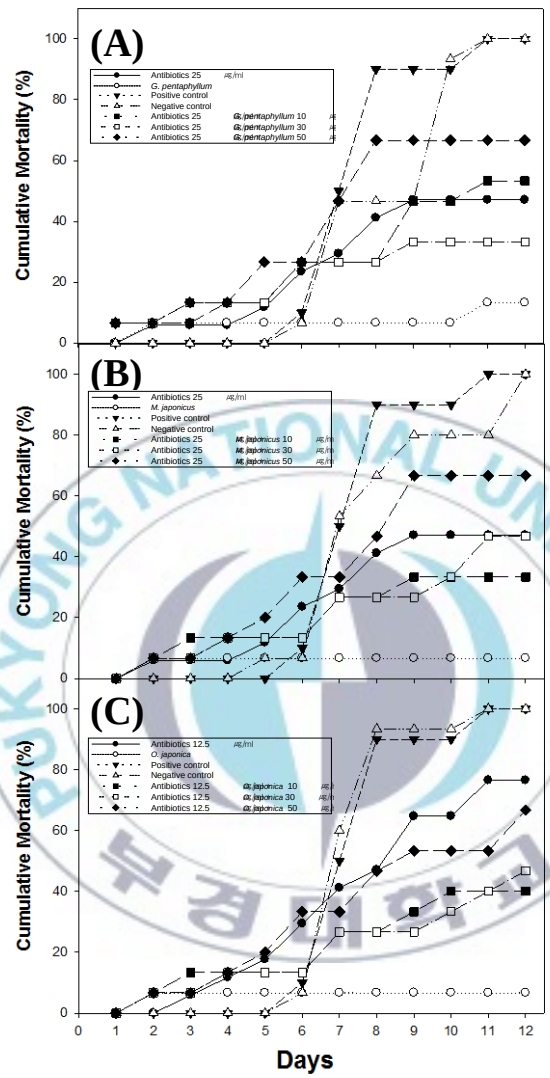

Fig. 5. The cumulative mortalities in olive flounder injected three natural plant compounds with antibiotics after the challenge of *E. tarda* at 22±1℃ (A) *G. pentaphyllum*, (B) *M. japonicas*, (C) *O. japonica*

The results of this study antibiotics and plant extracts with a mixture of reduce the mortality caused by pathogens *E. tarda* was found to increase persister cell formation by inhibiting the survival of flounder. Therefore, the three extracts used in the study of *G. pentaphyllum*, *M. japonicas* worry that does not have a concentration of the antimicrobial activity of antibiotics in combination with the persister cell reducing effect of it was confirmed that. To prevent residues in animals and the emergence of resistant strains of these results for the purpose of disease prevention and treatment of side effects, such as antibiotic usage dramatically, due to the increase of resistant bacteria and antibiotic residues in fish by reducing the incongruity of the host organisms, reduces result suggests the possibility of a new judgment. Future reduction persister cell extract and antibiotics by livestock or aquaculture industry and applied research should be conducted to clarify the mechanism of the effect is suggested.

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Ⅳ. CONCLUSION

High concentration of antibiotics has been used to treat the outbreak of dwardsiellosis caused by *Edwardsiella tarda* in aquaculture. However, not all of the bacteria have been killed with high concentrations of antibiotics treatment by the formation of persister cells with a dormant state. The main objective of this study was to kill persister cell using antibiotics with the addition of natural plant compounds. Antibiotics used in this study consist of 100 mg/ml florfenicol and 100 mg/ml amoxicillin. Ten natural plant compounds with persister cell inhibitor activity to *E. coli* were obtained from Protein Engineering and Systems Biology Lab. of Sungkyunkwan University. The persister cell inhibition activities of those natural plant compounds were evaluated in test tube. Concentrations of the antibiotics were in the ranges of 25~200 μg/ml. The persister cell formation was observed after 16 hours of culture. Persister cells were killed by antibiotics with natural plant compounds. Among ten natural plant compounds, *Gynostemma pentaphyllum*, *Mallotus japonicus*, and *Orixa japonica* showed persister cell formation inhibition activities. The optimal concentrations of *G. pentaphyllum*, *M. japonicus*, and *O. japonica* for the inhibitor of persister cell formation were 100 μg /ml, 100 μg /ml, and 200 μg /ml, respectively. *In vivo* study was carried out to evaluate the effect of the antibiotics with natural plant compounds using aquacultural fish, olive flounder, as test animals. *G. pentaphyllum*, *M. japonicus*, and *O. japonica* of 30 μg/ml, 10 μg/ml, and 10 μg/ml with antibiotics reduced

cumulative mortalities, showing the effectiveness of persister cell inhibition.

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