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Production of Anti-*Helicobacter pylori* Metabolite by the Lichen-Forming Fungus *Nephromopsis pallescens*

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The present study was conducted to evaluate the antibacterial activity of lichen-forming fungi (LFF) against *Helicobacter pylori*, and to optimize the culture conditions of LFF for maximum production of natural antibiotics against *H. pylori*. To accomplish this, a screening assay was first conducted among 19 species of LFF. The extract of *Nephromopsis pallescens* (KOLRI-040516) exhibited the strongest anti-*H. pylori* activity. Bioautograghic TLC and HPLC analysis identified usnic acid as the main antibacterial substance produced by *N. pallescens*. The growth of *N. pallescens* and production of antibacterial substances produced by the fungus were then investigated under several culture conditions including the culture media, initial medium pHs, incubation temperatures, and the degree of aeration. The results indicated that culture in MY medium with an initial pH of 6.0, a temperature of 15°C and a low degree of aeration supported the largest usnic acid production of the fungus (16.4 μg usnic acid/g dry biomass). Especially, aeration was found to be an important factor that affect both growth and usnic acid production of *N. pallescens*.

***Keywords*:** *Nephromopsis pallescens*, anti-*Helicobacter pylori* activity, usnic acid, culture conditions

Lichens produce numerous unique secondary metabolites with various bioactivities including antitumour, antibacterial, antifungal, antiviral, anti-inflammatory, and antioxidant activities (Oksanen, 2006). However, lichen substances have long been neglected by the pharmaceutical industry because of their slow growth in nature and difficulties in their artificial cultivation (Crittenden and Porter, 1991). In the past decade, methods for the isolation and culture of lichen symbionts have been established (Yamamoto *et al*., 1985), making it possible to produce bioactive lichen secondary metabolites by culturing lichen mycobionts in a sustainable way. Subsequently, several methods of bioactivity screening have been conducted using cultured lichens instead of wild lichen thallus (Behera *et al*., 2005).

Helicobacter pylori has been shown to be an essential etiological agent involved in gastritis, peptic ulcers, and gastric malignancy. Because of the prevalence of antibiotic-resistant *H. pylori* strains, it is necessary to search safe and effective agents for its treatment. Therefore, we screened the anti-*H. pylori* activity among the acetone extracts of 19 species of LFF, and found that LFF of *N. pallescens* has the most potent anti-*H. pylori* activity. However, the growth rate of this LFF was very slow and the production of the effective compounds was limited. In order to utilize this LFF as a potential producer of anti-*H. pylori* agents in the future, the production of the effective compounds should be improved. Factors stimulating fungal growth and the production of secondary metabolites such as media, temperature, pH, and different carbon and nitrogen sources have been discussed for several species of lichens (Yamamoto *et al*., 1987; Yamamoto *et al*., 1993; Behera *et al*., 2006). However, different LFF have different nutrient requirements and culture condition preferences, and there have been no such studies of *N. pallescens*. In addition, previous studies seldom evaluated the effect of culture aeration on the growth rate and production of the secondary metabolites of LFF. Therefore, in this study, we evaluated the effect of culture media, initial medium pH, incubation temperature and the degree of aeration to optimize the culture conditions to improve the growth rate and production of anti-*H. pylori* compounds of *N. pallescens* LFF.

Materials and Methods

Isolation and maintenance of the LFF

The LFF tested in this study were obtained from the Korean Lichen and Allied Bioresource Bank (KOLABIC), Sunchon National University, Korea. Isolation and confirmation by ITS sequence analysis of the LFF were previously described (Wei *et al*., 2008). Fungi were cultured on MY (malt-yeast extract) medium at 15°C in the dark.

Determination of the anti-*H. pylori* activity

The mycelia of each LFF were harvested after cultivation for two months. They were subsequently freeze-dried and extracted by acetone (100 mg dry weight/ml acetone). The acetone extracts were then filtered using Whatman filter paper No.1 (Whatman Ltd., UK) and were used as sample solutions in the anti-bacterial assay.

H. pylori strain No. 26695 was obtained from the *H. pylori* Korean Type Culture Collection (HPKTCC). The bacterial cells were microaerobically incubated (85% N_2, 10% CO_2, 6% O_2) on BHIA (brain heart infusion agar) supplemented with 5% horse blood at 37°C in GasPak® Jars (BBL Microbiology Systems, USA) using an Oxoid

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gas-generating kit (Oxoid Limited, England).

The anti-*H. pylori* activity of LFF extracts was detected by disk diffusion method. Air-dried 8 mm paper disks charged with 200 μl of the sample solution (or 200 μl of acetone used as negative control) were placed on agar plates that were seeded with the *H. pylori.* And the diameters of the inhibition zones were determined after 48 h incubation at 37°C under mircroaerophilic conditions. The experiments were run in triplicate, and the results are presented as the Mean±SD of three independent experiments.

Culture of the LFF under different conditions

The LFF of *Nephromopsis pallescens* (KOLRI-040516) with the strongest anti-*H. pylori* activity was subcultured under different conditions to optimize the culture condition for growth rate and production of the anti-*H. pylori* metabolites. The LFF mycelium from MY agar plates were collected and gently crushed using a sterile mortar and pestle for subsequent use as inoculums. The effect of different basal media were evaluated by inoculating the ground mycelium in six different media: MY (malt-yeast extract), MYM (MY+2% mannitol), MYR (MY+2% ribitol), S2% (Sabouraud+2% glucose), MS (Murashige and Skoog medium), and PDB (potato dextrose broth). The effect of pH were studied by varying the incubation pH from 3.0 to 9.0. The effect of temperature were studied by varying the incubation temperatures from 10°C to 25°C. Ground mycelium were inoculated into 50 ml of liquid medium (for pH and temperature analysis, MY media was used) for each treatment, and incubation was conducted in 250 ml conical flasks on a rotary shaker at 150 rpm without light. To evaluate the effect of aeration degree, the ground mycelia were inoculated into a bioreactor (20 L) with a continuous air supply (10 L/min). The same amount of LFF was inoculated in a shaking incubator (150 rpm) without air supply. Both samples were then cultured in the same volume of liquid MY medium (pH 6.0) at 15°C without light.

Determination of the growth rate and the production of the anti-*H. pylori* metabolites

After two-month culture, the fresh mycelia were harvested by removing the culture medium by filtration with a filter paper No. 1 (Whatman Ltd., UK). The dry weight (DW) was measured after freeze-drying and the relative growth rate (RGR=DW of harvested mycelium/estimated DW of inoculated mycelium; estimated DW almost equal to one sixth of the fresh weight) was calculated.

To determinate the production of the anti-*H. pylori* metabolites, 20 ml of liquid medium was sampled for each liquid culture. The media were then freeze dried, after which the residues were extracted with 1 ml of acetone. The acetone extracts of each culture media were then tested for an anti-*H. pylori* activity assay as mentioned above.

In all cases, three repeat experiments were performed; results are presented as the Mean±SD of three independent experiments.

Identification of the effective compound

Bioautograghic TLC: The sample solutions obtained as described above was analyzed by TLC on silica gel plates using the solvent system toluene-acetic acid (170:30, v:v). TLC plates were developed in duplicate and one set was used as the reference chromatogram. Spots and bands were visualized by UV light and by spraying with 10% sulfuric acid. The other set was sterilized under UV light for 20 min and then gently placed face down on BHIA medium so that the silica layer was in contact with the inoculated medium. The medium covered by the TLC plate was then incubated under the mircroaerophilic conditions described above for 24 h. The location of the zone of inhibition indicated the location of the active compounds on the TLC plate.

HPLC analysis of the anti-*H. pylori* secondary metabolites: Bioautographic TLC plates were used as a reference to locate anti-*H. pylori* active compounds on prep-TLC plates developed in the same solvent. Silica gel at the active spot area was collected, dissolved in acetone, and filtered. The solution was then analyzed by HPLC (LC-10AT, Shimadzu, Japan) under the following conditions: YMC-Pack ODS-A S-5 μm 150×4.6 mm I.D. column; solvent, methanol: H_2O: H_3PO_4 (80:20:1, v:v); 1 ml/min flow rate; photodiode array detector (range 180-700 nm); detecting wavelength, 254 nm for HPLC and 180-400 nm for UV-spectrum analysis; temperature, 40°C. The lichen substance was identified by comparing its retention times and UV-spectra with the database of the authentic substances in the Laboratory of Advanced Bio-Production Science at Akita Prefecture University, Japan as previously described (Luo *et al*., 2009).

Results

Anti-*H. pylori* activity of LFF extracts

Among the tested 19 species of LFF, *N. pallescens* exhibited the strongest anti-*H. pylori* activity (Table 1). The inhibition zone (15.80 mm) was comparable with that of ampicillin and erythromycin (10 μg/ml) as positive controls. The extract of *Parmelia laevior*, *Ramalina yasudae*, and *N. asahinae* also showed moderate inhibition activity against *H. pylori*, while the other LFF showed no activity.

Bioautographic TLC and HPLC

Bioautographic TLC showed one zone of inhibition against

Table 1. The anti-*Helicobacter pylori* activity of LFF extracts

Species of LFF	Inhibition zone (mm)[a]
Canomaculina subtinctoria	-
Cetrariopsis wallichiana	-
Cladonia macilenta	-
Cladonia metacorallifera	-
Cladonia rangiferina	-
Everniastrum cirrhatum	-
Hypogymnia hypotrypella	-
Melanelia olivacea	-
Myelochroa aurulenta	-
Myelochroa entotheiochroa	-
Myelochroa irrugans	-
Nephroma helveticum	-
Nephromopsis asahinae	8.7±2.1
Nephromopsis pallescens	15.8±1.5
Parmelia adaugescens	-
Parmelia laevior	9.3±0.8
Ramalina conduplicans	-
Ramalina sinensis	-
Ramalina yasudae	9.1±1.2
Ampicillin (10 μg/ml)[b]	14.7±0.5
Erythromycin (10 μg/ml)[b]	17.3±1.4

[a] All the data presented are the Mean±SD of three independent experiments.
[b] Positive controls
- No inhibition

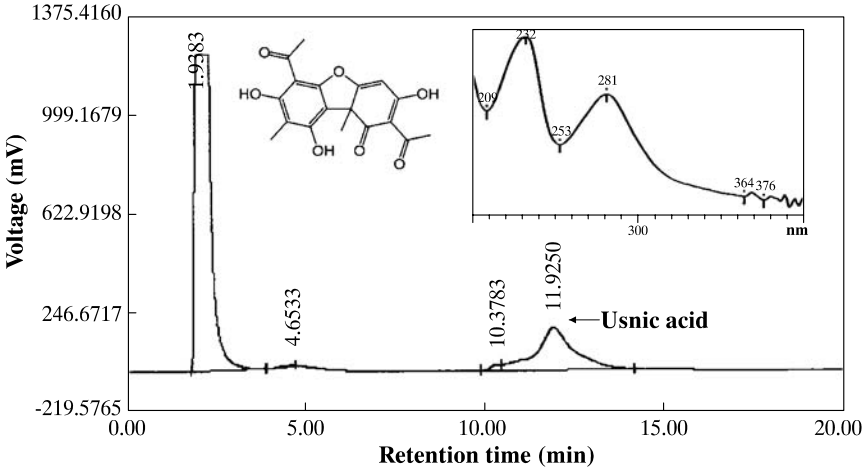

Fig. 1. The HPLC chromatograms of the anti-*H. pylori* active compounds isolated from the acetone extract of *N. pallescens* LFF culture medium. (*N. pallescens* LFF was cultured in a shaking flask with MY medium at 15°C for 2 months). The peak with a retention time of 11.925 min is usnic acid. The insets present the molecular structure and the UV spectrum of usnic acid.

H. pylori, indicating the presence of active compound produced by *N. pallescens* LFF. The compound showing antibacterial activity was identified as usnic acid based on comparison of the Rf value on TLC and the retention time of HPLC with an authentic standard (Fig. 1).

Growth rate and the production of the anti-*H. pylori* metabolites of *N. pallescens* under several culture conditions

Effect of culture media: The effect of culture media on the total growth of *N. pallescens* mycobionts is presented in Fig. 2. MYM medium enriched with mannitol resulted in the optimal growth rate (RGR=8.83), although MYR and MS media also supported a rapid growth rate for this fungi (RGR=7.85 and 7.74, respectively). The growth rate of the fungus on MYR and MS media was about two times the growth rate on MY medium that did not contain a supplemental carbon source. S2% medium is commonly used for the culture of LFF; however, *N. pallescens* mycobiont grows very slowly on this media (Fig. 1). The extract of MY medium showed the strongest anti-*H. pylori* activity, while the extract of MYM and MYR showed moderate inhibition, and no activity was detected from the extract of MS, PDB, and S2% cultures.

Effect of incubation temperature and initial medium pH: The effect of temperature on the growth rate and anti-*H. pylori* activity of the culture are shown in Fig. 3. *N. pallescens* exhibited the fastest growth rate (RGR=8.04) and the strongest anti-*H. pylori* activity at 10°C. Both the growth rate and anti-*H. pylori* activity decreased as the culture temperature increased (Fig. 3).

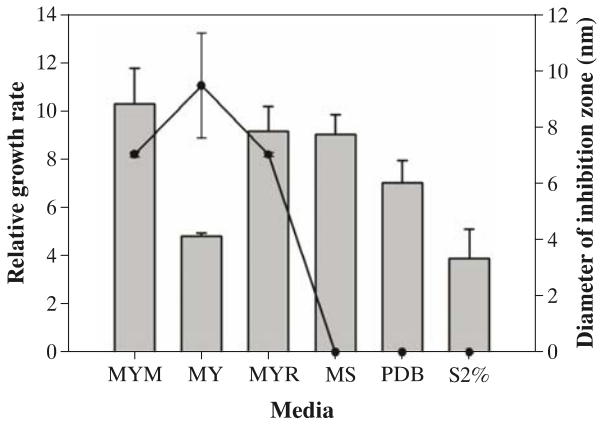

Fig. 2. The effect of different media on the growth and anti-*H. pylori* activity of *N. pallescens* LFF. MYM was the optimal medium for growth while MY without any extra carbon source was the optimal medium for the production of anti-*H. pylori* compounds. The bars indicate the relative growth rate; the line indicates diameters of the inhibition zones of the extracts on the *H. pylori* plate.

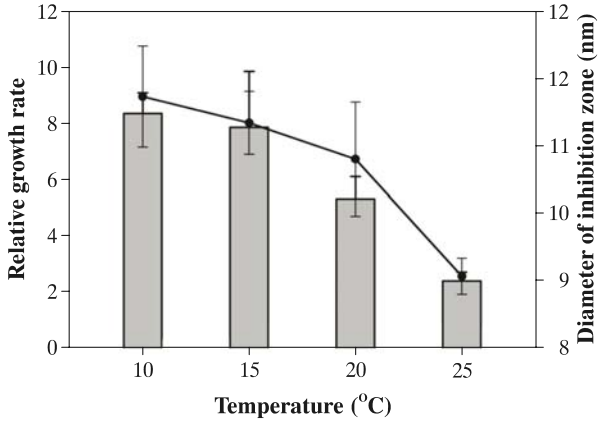

Fig. 3. The effect of temperature on the growth and anti-*H. pylori* activity of *N. pallescens* LFF. A temperature of 10°C supported the fastest growth and the most potent anti-*H. pylori* activity of this LFF, and the growth rate decreased with increasing culture temperature. The bars indicate the relative growth rate; the line indicates diameters of the inhibition zones of the extracts on the *H. pylori* plate.

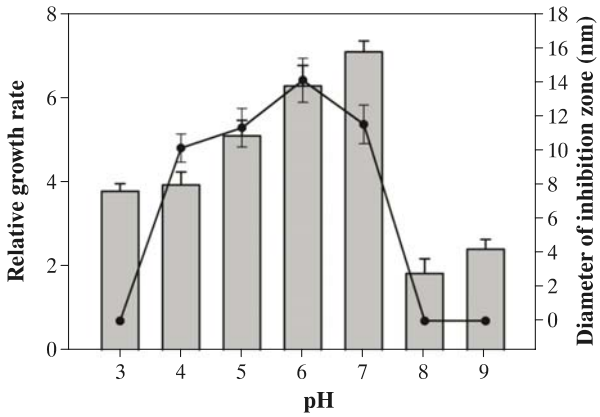

Fig. 4. The effect of initial medium pH on the growth and anti-*H. pylori* activity of *N. pallescens* LFF. pH 7.0 was the optimal initial pH for the growth of this LFF, while pH 6.0 was the optimal initial pH for the production of anti-*H. pylori* compounds. The bars indicate the relative growth rate; the line indicates diameters of the inhibition zones of the extracts on the *H. pylori* plate.

The effect of the initial medium pH are shown in Fig. 4. The optimal initial medium pH for the growth of *N. pallescens* LFF was observed at pH 7.0 (RGR=7.14); however, the production of anti-*H. pylori* active compounds was best at pH 6.0. The fungus showed very slow growth and no anti-*H. pylori* activity under alkaline conditions, which likely reflects the neutral to acidic nature of the habitats of this lichen.

Effect of culture aeration: After two-month culture of *N. pallescens* LFF in an air floating bioreactor and a shaking incubator, the amount of mycelium and the color of liquid media differed significantly between the cultures. The mycelium pellets in the air floating bioreactor were about five mm in diameter and tightly entangled, and the color of the medium was still the same yellow of the initial MY medium. Conversely, in the submerged flask cultured in the shaking incubator, the mycelium pellets were about 3 mm in diameter and not connected, and the color of the liquid medium was dark brown, indicating that more secondary metabolites were produced by this culture method. The effect of culture aeration on the growth rate and secondary metabolic capacity of *N. pallescens* mycobiont were quantified and summarized in Table 2. The results suggested that the supply of air led to a significant increase in the growth rate of LFF, with the level of RGR in the air floating system being about one fold greater than that of the submerged culture. However, the supply of air significantly inhibited the secondary metabolic capacity, the production of usnic acid by the shaking flask culture (16.4 μg/g dry biomass) sharply reduced to 0.2 μg/g dry biomass by the air-floating culture.

Table 2. Effects of the degree of culture aeration on the growth rate and usnic acid production of *Nephromopsis pallescens* (KOLRI-040516) mycobiont

Culture methods	Relative growth rate	Usnic acid production (μg/g dry biomass)
No aeration (Shaking flask culture)	8.2±0.5	16.4±2.1
Forced aeration (10 L/min) (Air floating bioreactor)	15.5±1.6	0.2±0.0

All the data presented are the Mean±SD of three independent experiments.

Discussion

Usnic acid is uniquely found in lichens, since its first isolation in 1884, numerous biological activities such as antibacterial, antiviral, antiprotozoal, antiproliferative, anti-inflammatory, and analgesic activity have been reported (Ingolfsdottir, 2002). The antibacterial activity of usnic acid is most well-known. However, it is interesting to note that, the antibacterial activity of usnic acid seemed to be restricted to the Gram-positive bacteria; few reports mentioned its antibacterial activity against Gram-negative bacteria. In present study, usnic acid produced by *N. pallescens* was found to have potent inhibitory activity against Gram-negative bacteria *H. pylori*, and the production of usnic acid by *N. pallescens* under the optimum condition (16.4 μg/g dry biomass) was found to be higher than that of *Usnea ghattensis* (3.9 μg/g dry biomass) in previous study (Behera *et al*., 2006).

Because of the great potential for medicinal and ecological applications, the production of usnic acid by lichen mycobionts had been studied in recent years. Previous studies described several factors that could influence the production of usnic acid. For example, Kinoshita *et al*. (2001) suggested that nitrogen resource had a significant influence on usnic acid production of a mycobiont of *Usnea hirta* in liquid medium. Moreover, Behera *et al*. (2006) indicated that high osmotic pressure of culture medium increased the production of usnic acid of *U. ghattensis* mycobiont.

In the present study, we found that all of the tested factors, including media, temperatures, initial medium pH, and the supply of air, significantly affected the fungal growth rate and usnic acid production, but that these factors did not always have the same effect on both grow rate and usnic acid production. For instance, adding extra mannitol and ribitol increased the growth rate of *N. pallescens* LFF on MY medium by about one-fold; however, on the contrary, the addition of mannitol and ribitol inhibited the usnic acid production. A similar phenomenon was also observed when culture of the LFF in an air floating bioreactor was conducted. The dry weight of the mycelium was doubled by supplying continuous filtered air, while the production of usnic acid was reduced by about 80 times. This may have occurred because when more resources are used to produce the biomass, fewer resources could be used to produce the secondary metabolites. Previous study mentioned that LFF are organisms that prefer a high degree of aeration (Bloomer, 1968); therefore, the supply of continuous air increased the growth of *N. pallescens* mycobiont. In contrast, the submerged culture without enforced-air supply could be a stress condition, which caused fungi to sacrifice growth to produce usnic acid in response to the stress. Although the exact recipes and conditions that trigger LFF to produce secondary metabolites are not known, it is known that ecological factors, including physiological stress, are necessary for the induction of secondary metabolic pathways and the production of specific lichen substances (Stocker-Wörgötter, 2001). In present study we found that the culture aeration might

be one of these factors, and LFF *N. pallescens* prefers low degree of aeration to produce usnic acid. In future studies, more stress conditions should be considered for promotion of the production of secondary metabolites.

In conclusion, the results of our experiment suggested that 1) acetone extract of LFF *N. pallescens* exhibites strong anti-*H. pylori* activity 2) usnic acid is the main effective anti-*H. pylori* metabolite produced by *N. pallescens* 3) culture in MY medium, initial pH 6.0 and low degree of aeration was found to be the optimum culture condition for usnic acid production by *N. pallescens*, giving a production of 16.4 μg usnic acid/g dry biomass. Considering the strong anti-*H. pylori* activity of usnic acid and the high production of usnic acid by LFF of *N. pallescens,* this LFF can be used as a potential producer of anti-*H. pylori* agents in the future.

Acknowledgements

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