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Antimicrobial Protein Produced by a Novel Endophyte DNN6 Isolated from *Laminaria japonica* Aresch

Fuyun Zhang, Ying Zhang, Feng Xu, Yang Yang and Yu Zhao

Dalian Ocean University, College of Food Science and Engineering, China.

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The aim of this work was to produce antimicrobial protein using novel endophyte DNN6. The endophyte DNN6 that showed distinct antibacterial activity was isolated from *Laminaria japonica* Aresch in Dalian China. The strain DNN6 was identified as *Bacillus licheniformis*. The orthogonal matrix method was used to optimize the culture conditions for antibacterial activity of strain DNN6, and the optimum conditions for DNN6 were cultivated in 50 g/L sucrose and 10 g/L peptone with pH 9 at 25°C for 6 d. To obtain the antibacterial protein, different saturation of ammonium sulfate was used. The high yield (2.866 g/L) and antibacterial activity of protein from strain DNN6 was precipitated by 95% saturation of ammonium sulfate. Furthermore, the antifungal activity was detected, and this protein demonstrated some degree of inhibition to all the 10 funguses. The protein of strain DNN6 is potential for application as an antimicrobial substance.

Key words: Endophyte; Isolation; Antimicrobial protein; Optimization; Production .

Endophytes reside inside plant tissues, without causing any apparent or detectable symptoms to the plant[1,2], and the existence of endophytic microorganisms may play a significant role for plant health and plant protection[3-5]. Research results indicate that the endophytic microogonisim could produce bioactivity metabolites such as taxol[6], vincamine[7], enzymes, antibiotics and siderophores[8-11]. The endophytic *actinomycetes* and *Streptomyces* sp. obtained from snake-vine have been repoted to produce novel peptide antibiotics, and those peptides possessed activities against several pathogenic fungi and bacteria[12-14]. Antibiotic active bacteria associated with brown alga has also been reported[15], but the endophytic and epiphytic microorganisms were not differentiated. The activity metabolites of these strains have neither been definite. Therefore, in this study, endophytes from *Laminaria Japonica* Aresch were isolated. The endophyte DNN6 with antibacterial activity was characterized, and the antibacterial protein of it was precipitated by ammonium sulfate. In vitro antagonistic activity test against variety of fungi was also performed to evaluate the potential of this protein.

* To whom all correspondence should be addressed.
Tel.: 86-411-84762889, Fax: 86-411-84763508;
E-mail: zhangfuyun2004@126.com

METHODS

Chemicals and indicator microorganisms

Chemicals used in the microbiological culture media, including beef extract, peptone and agar were purchased from BEIJING AOBOXING BIO-TECH CO., LTD. Other chemicals used in this study including ammonium sulfate, acrylamide, N, N'-methylenebis-2-Propenamide and ethyl alcohol were analytical grade.

The *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Staphylococcus aureus* ATCC 6538, *Clostridium perfringens* ATCC 13124 and *Bacillus subtilis* ATCC 11562 were used for screening antibacterial activity. These strains were provided by Dalian institute of chemical physics, Chinese academy of sciences.

Isolation of endophytes from *Laminaria Japonica* Aresch

Laminaria japonica Aresch samples were randomly collected from the seafloor of Heishijiao Bay, located at Dalian, the southern part of Liaoning province (China), in April 2010. The healthy samples were placed in sterile plastic bags, which were taken to the laboratory and processed within 10 min. The roots and leaves of kelp were washed 5 times with sterile seawater, rinsed in 70% ethanol for 90 seconds and in 0.1% mercuric chloride for 18 seconds. Samples were washed in sterile water 7 times to remove the sterilization agents before being grounded. Each sample was cut into small pieces and grounded into uniform slurry. The slurry was inoculated on Rose Bengal Agar, Nutrient Agar, Gauze's Medium No.1 medium dissolved in water or seawater by spread plate technique, and some surface sterile leaf pieces were plated on the same medium to check the efficacy of surface sterilization. The plates were incubated at 28°C for 10 days and were observed every day. The emerging colonies were purified by streaked plate method.

Detection of antibacterial activity of endophytes

The antibacterial activity of the isolate was tested by using disc diffusion method[16] with minor modifications. The indicators were inoculated in Beef Extract and Peptone medium and incubated at 37°C. 100 µL overnight culture of indicator bacteria strains were inoculated on Nutrient Agar by spread plate respectively, and the sterile filter paper discs (6 mm) were placed on the surface of Nutrient Agar. The endophytes were cultured at 30°C for 96 h in liquid medium, centrifuged at 4000 g for 10 min without filtration. 10 µL supernatant sample was dropped on the filter paper. The no inoculated medium was used as positive control and 75% ethanol was used as positive control. All plates were incubated at 37°C for 2 days.

Identification of endophyte DNN6

The strain DNN6 was identified according to its morphological, physiological and biochemical characters by using standard manuals[17]. The 16S rDNA gene of strain was also analysised. The preparation of genomic DNA, amplification and sequencing of 16S rDNA was carried out by TaKaRa Biotechnology Co. Ltd., China. The 16S rDNA sequence of strain DNN6 was compared with the sequences in the GenBank nucleotide library by BLAST search through the National Center for Biotechnology Information (NCBI). Multiple alignments were done with CLUSTALX 1.8.1. The MEGA4.0 software package was used to analyze the Phylogenesis. The neighbor-joining method[18] was used to build unrooted tree.

Optimization of culture conditions for antibacterial activity

The Sabouraud Dextrose Medium, Sabouraud Sucrose Medium, Beef Extract Peptone Medium and King'A Medium were used to select the better medium for antibacterial activity. The antibacterial activity was detected after 96 h of incubation at 30°C.

Based the above results, the Sabouraud's Sucrose Medium was used as the basal medium for optimization. The culture conditions were optimized using the orthogonal matrix method. The orthogonal test $L_{16}(4^5)$ is listed in Table 1.

Extraction of antibacterial protein

The Bacteria Strain DNN6 was inoculated in the optimal medium (sucrose, 50 g; peptone, 10 g, water, 1000 mL, pH 9), and incubated on a rotary shaker shaking at 150 rpm and 25°C for 6 days. The fermentation was centrifuged at 4000 g and 4°C for 10 min. Then the ammonium sulfate was added to 30 mL supernatant and kept at 4°C for 12 h. The saturation of ammonium sulfate is 70%, 75%, 80%, 85%, 90%and 95% respectively. The mixtures were centrifuged at 10000 g for 30 min. The sediments were re-dissolved and dialyzed 3-4 days to remove the ammonium sulfate. Finally, crude protein samples were obtained by freeze-drying method. The protein productivity and antibacterial activity was compared. The protein with highest productivity and antibacterial activity was analyzed by SDS-PAGE with 4% stacking gel and 10 separating gel[19].

Antifungal activity

The antifungal activity of protein was detected by Oxford Cup method[20]. The pathogenic fungi *Gerlachia nivalis* (*Ces*, ex *Sacc*.), *Fusarium oxysporum* Schlecht, *Ralstonia solanacearum*

(Burkholderia, Pseudomonas), *Botrytis cinerea* Pers, *Fusarium moniliformis* intermedium Neish & M. Legg, *Fusarium oxysporum* f sp. *Melonis* (muskmelon), *Trichothecium roseum* (Bull.) Link, *Alternaria solani* Sorauer, *Mucor mucedo* (L.) Brefeld, VD-8 of *Verticillium dahliae* (provided by Dalian institute of chemical physics, Chinese academy of sciences), were cultured in Sabouraud's Sucrose Medium at 28°C for 4 days. The test plates were prepared with Sabouraud Agar and inoculated with 100 mL fungi fermentation by spread plate method. Five Oxford cups were spaced on the agar surface of each petri dish. The 150 µL protein samples (500, 1000 and 1500 µg/mL) and positive control (4 µL/ mL benziothiazolinone) were pipetted in Oxford cup respectively. The inhibition zone was evaluated after incubation at 28°C in the darkness for 120 h.

Statistical analysis

All experiments were performed in triplicates. Statistics of date was conducted by using statistical package in the software program Microsoft Excel 2010. Data are presented as means ± SD. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Isolation and detection antibacterial activity of the endophytes

In total, 26 endophytes were obtained from healthy *Laminaria japonica* Aresch collected from the seafloor of Heishijiao Bay, located at Dalian in China according to their morphological features. Among these endophytes, 12 strains were isolated on Nutrient Agar dissolved in water, 6 strains were isolated on Nutrient Agar dissolved in seawater, 3 strains cultivated on the Rose Bengal Agar dissolved in water, and 5 on the Rose Bengal Agar dissolved in seawater. No growth was detected on the Gauze's Medium No.1 medium.

The antibacterial activity of endouphytes from plant has been reported before [21]. To screen the antibacterial activity of endouphytes from *Laminaria japonica* Aresch, the disc diffusion method was used. The results of antibacterial activity of the endophytes against five bacteria are shown in Table 2.

All isolates except for 8 strains showed antibacterial activity to indicator bacteria. Some isolates such as, DNN6, DNN7, DNN8 and HSN2 showed higher antibacterial activity and broad antibacterial spectrum. Among the isolates, DNN6 was selected for further analysis because the antibacterial activity of its fermentation broth was highest when the cells were removed.

Identification of endophyte DNN6

The colony characteristics of endophyte DNN6 were observed and described (Fig.1.). The colonies of it are circular, entire, convex, wrinkle, opaque, brittle and white. The endophyte DNN6 with spore belongs to bacillus. Cell width ranges from 0.5 to 1 µm, and cell length ranges from 3.0 to 3.5 µm. The Gram staining, catalase, hydrolysis of starch, casein hydrolysis, gelatin hydrolysis, nitrate reduction, Voges-Proskauer test, and litmus milk test reactions are positive. The strain can utilize sodium citrate. It can also ferment xylose, glucose, mannose, arabinose, but cannot produce lecithase. The strain can grow in 8% (w/v) NaCl, 0.02% (w/v) sodium azide, and the acidic nutrient broth of pH 5.7 respectively. The highest temprature for strain DNN6 growth is 53°C. These results were in general agreement with *Bacillus licheniformis*[22]. The result of BLAST showed the identities of the 16S rDNA sequence between strain DNN6 (GenBank accession number: KF574817) and KIBGE-IB1 (GenBank accession number: Gu216258) was 99%. In the phylogenetic tree, that strain DNN6 and KIBGE-IB1 belong to the *Bacillus* fimily (Fig.2.). The branchs of DNN6 and KIBGE-IB1 are sister

Table 1. Factors and levels for orthogonal test $L_{16}(4^5)$

Level	Factors				
	(A)Temperature (°C)	(B) pH	(C)Time (h)	(D)Sucrose (g/L)	(E)Peptone (g/L)
1	25	3.3	72	20	2
2	30	5.3	96	30	6
3	35	7.3	120	40	10
4	40	9.3	144	50	14

Table 2. Antibacterial activity of endophytes against 5 indicator bacteria by disc diffusion method

Isolates	Indicator bacteria				
	P. vulgaris	*B. Subtilis*	*E. Coli*	*C. Perfringens*	*S. Aureus*
Positive control	+++	+++	+++	+++	+++
Negative control	—	—	—	—	—
DNN2	+++	—	+	—	—
DNN3	+++	—	++	—	—
DNN4	++++		++		
DNN5	++		++		+
DNN6	+++	++	+++	—	+++
DNN7	+++	+++	+++	—	++
DNN8	++++	+++	++	—	+++
HNN1	+	—	++	—	—
HNN2	—	—	++	+	—
HNN 3	+++	++	++	—	++
HNN4	++	++	+++	—	++
HNN5	+	—	++	—	—
HNN6	—	+	++	—	—
DSN3	—	+	++	—	—
HSN1	—	—	++	+	—
HSN2	+++	++++	+++		++++
HSN3	—	++	—	—	++
HSN5	+++	—	—	—	—

DNN: Isolates on Nutrient Agar dissolved in water, HNN: Isolates on Nutrient Agar dissolved in sea water, DSN: Isolates on Rose Bengal Agar dissolved in water, HSN: Isolates on Rose Bengal Agar dissolved in sea water, P. vulgaris: *Proteus vulgaris*, B. Subtilis: *Bacillus subtilis*, E. Coli: *Escherichia coli*, C. Perfringens: *Clostridium perfringens*, S. Aureus: *Staphylococcus aureus.*, +: d≤8 mm, ++: 8 mm<d≤9 mm, +++: 9 mm<d≤10 mm, ++++: d>10 mm, (-): no inhibition

Table 3. The design and results of orthogonal test $L_{16}(4^5)$ for the antibacterial activity of strain DNN6

Test	Parameters					Indicators (Diameter of inhibition zone / mm)				
no.	A	B	C	D	E	*E. Coli*	*B. Subtilis*	*P. vulgaris*	*C. Perfringens*	*S. Aureus*
1	A_1	B_1	C_1	D_1	E_1	30	22.5	19.5	14	15
2	A_1	B_2	C_2	D_2	E_2	40	24.5	25	12	36
3	A_1	B_3	C_3	D_3	E_3	40	28.5	26	15	30
4	A_1	B_4	C_4	D_4	E_4	39	35.5	29	19	34
5	A_2	B_1	C_2	D_3	E_4	12	14	16	14	14
6	A_2	B_2	C_1	D_4	E_3	24	32.5	25.5	18	26
7	A_2	B_3	C_4	D_1	E_2	33	26.5	27	15	29
8	A_2	B_4	C_3	D_2	E_1	33	30.5	23	14	35
9	A_3	B_1	C_3	D_4	E_2	24	22	25	12	15
10	A_3	B_2	C_4	D_3	E_1	16	33	26	15	14
11	A_3	B_3	C_1	D_2	E_4	12	33.5	26	12	12
12	A_3	B_4	C_2	D_1	E_3	14	32	26	12	14
13	A_4	B_1	C_4	D_2	E_3	14.5	25	20	14	17
14	A_4	B_2	C_3	D_1	E_4	14	25	26	12	13
15	A_4	B_3	C_2	D_4	E_1	16	26	26	14	14
16	A_4	B_4	C_1	D_3	E_2	12	38	18	18	12

E. Coli: *Escherichia coli*, B. Subtilis: *Bacillus subtilis*, P. vulgaris: *Proteus vulgaris*, C. Perfringens: *Clostridium perfringens*, S. Aureus: *Staphylococcus aureus.*

Table 4. Analysis of $L_{16}(4^5)$ test for the antibacterial activity of strain DNN6

	E. Coli					*B. Subtilis*					*P. vulgaris*					*C. Perfringens*					*S. Aureus*				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
K_1	149	80.5	78	91.5	95	111	83.5	126.5	106	112	99.5	80.5	89	98.5	94.5	60	54	62	53	57	115	61	65	71	78
K_2	102	94	78	100	109	103.5	115	96.5	113.5	111	91.5	102.5	93	94	95	61	57	52	52	57	104	89	78	100	92
K_3	66	101	103	84	92.5	120.5	114.5	106	113.5	118	103	105	100	86	97.5	51	56	53	62	59	55	85	93	70	87
K_4	56.5	98	102.5	103	77	114	136	120	116	108	90	96	102	105.5	97	58	63	63	63	57	56	95	94	89	73
k_1	37.25	20.16	19.5	22.86	23.75	27.75	20.87	531.62	526.5	28	24.86	20.12	522.25	24.66	23.66	15	13.5	15.5	13.25	14.25	28.75	15.25	16.25	17.75	19.5
k_2	25.5	23.5	19.5	25	27.25	25.87	528.75	24.12	528.36	27.75	22.86	25.62	523.25	23.5	23.75	15.25	14.25	13	13	14.25	26	22.25	19.5	25	23
k_3	16.5	25.25	25.75	21	23.16	30.12	528.62	526.5	28.36	29.5	25.75	26.25	25	21.5	24.38	12.75	14	13.25	15.5	14.75	13.75	21.25	23.25	17.5	21.75
k_4	14.16	24.5	25.62	525.75	19.25	28.5	34	30	29	27	22.5	24	25.5	26.38	24.25	14.5	15.75	15.75	15.75	14.25	14	23.75	23.5	22.25	18.25
R	23.125	5.125	6.25	4.75	8	4.25	13.125	7.5	2.5	2.5	3.25	6.125	3.25	4.875	0.75	2.5	2.25	2.75	2.75	0.5	15	8.5	7.25	7.5	4.75
Optimal level	AECBD $A_1E_2C_3B_2D_4$					BCADE $B_4C_1A_3D_4E_3$					BDACE $B_3D_4A_3C_4E_3$					CDABE $C_4D_4A_2B_4E_3$					ABDCE $A_1B_4D_2C_4E_2$				

a $K_i^A = \Sigma$ the diameter of inhibition zone against target bacterium at *Ai*; b $k_i^A = K_i^A/4$; c $R_i^A = \max\{k_i^A\} - \min\{k_i^A\}$; E. Coli: *Escherichia coli*, B. Subtilis: *Bacillus subtilis*, P. vulgaris: *Proteus vulgaris*, C. Perfringens: *Clostridium perfringens*, S. Aureus: *Staphylococcus aureus*

group relationship, and the strain KIBGE-IB1 had been identified as *Bacillus licheniformis*. According the phylogenetic analysis, morphological, physiological and biochemical characters, the strain DNN6 should be identified as *B. licheniformis*.

Optimization of culture conditions

The research results revealed that different culture medium affected the antibacterial activity of strain DNN6 significantly (Fig.3.). The maximum diameter of inhibition zone (21 mm) against B. *subtilis* was observed when the strain was cultured in Sabouraud Sucrose Medium. The diameter of inhibition zone against others indicator bacteria of this fermented broth were also greatest. Therefore, this medium was selected as the basal medium for further experiments.

Since various parameters potentially affect the production of antibacterial substance, the optimization of culture conditions is critical to obtain more effective antibacterial substance. The orthogonal method enables different factors to be investigated simultaneously at several levels[23-25]. The orthogonal test $L_{16}(4^5)$ was used to optimize the culture conditions. The temperature, pH, time, the concentration of sucrose and peptone were designed at four levels based on the aforementioned results. The results of antibacterial activity for each fermentation test presented in Table 3 indicated that the diameter of maximum inhibition zone against 5 indicators was 40, 38, 29, 19 and 36 mm respectively. The inhibition zones against *Clostridium perfringens* were smallest in almost each test, and the great inhibition differences among each set of culture conditions were obvious. However, the best conditions cannot be select only based on these dates in Table 3, and an orthogonal analysis was further conducted. Thus, the K, k and R values were calculated and listed in Table 4.

The results in Table 4 exhibit that the influence of factors on the antibacterial activity was different to each indicator according to the R values. The influence decreased in the order as A>E>C>B>D, B>C>A>D>E, B>D>A>C>E, C>D>A>B>E and A>B>D>C>E respectively, however the influence of parameters decreases in the order A>B>C>D>E according to the overall rating of R values. The incubation temperature is the most important determinant of the bioactivity.

The diameter of inhibition zone against *Escherichia coli* increased from 12 to 40 mm as the temperature decreased from 40 to 25°C. The inhibition zone against *Staphylococcus aureus* also changes sharply as the temperature decreased. The pH value also affects the inhibition zone. The diameter of inhibition zone against *Bacillus subtilis* and *Proteus vulgaris* appears to show an increase

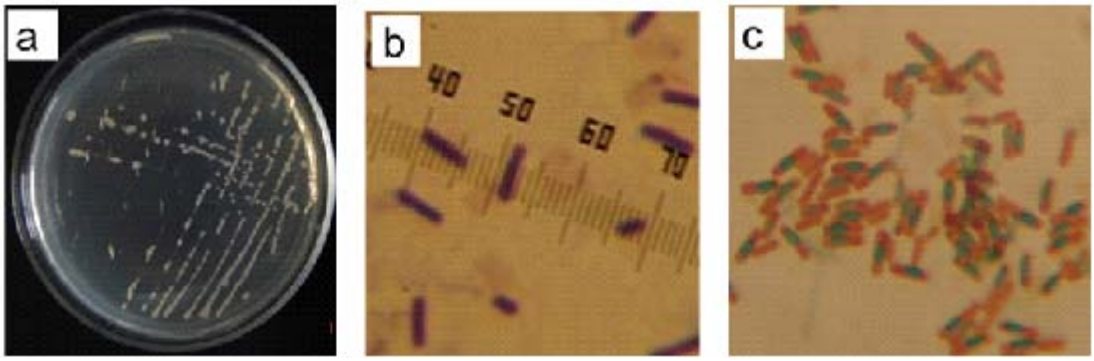

Fig. 1. The morphological character of endophyte DNN6. a: Colony; b: Gram staining; c: Spore staining

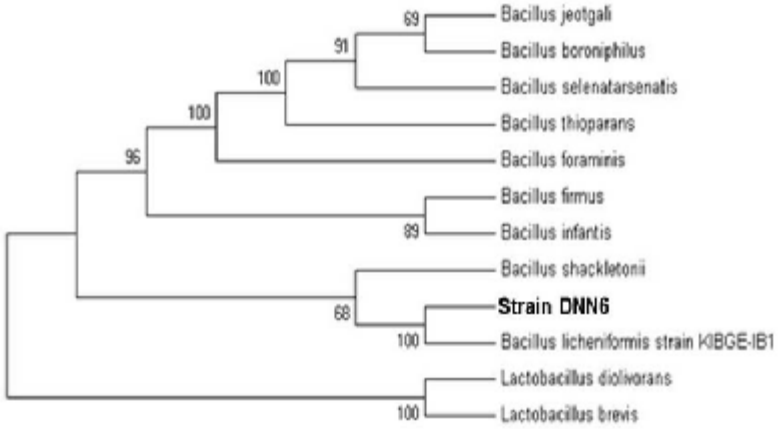

Fig.2 Phylogenetic tree based on the 16S rDNA sequence of strain DNN6 and related strains. The phylogenetic tree was constructed using neighbor joining algorithm in MEGA4.0 software. Numbers at the nodes indicate the bootstrap values from neighborhood-joining analysis of 100 replicates

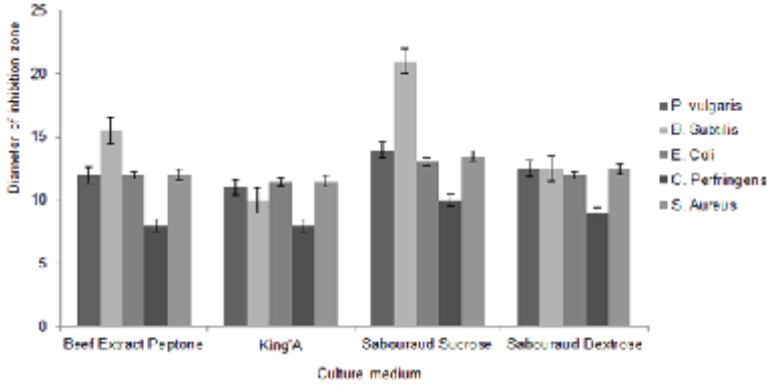

Fig. 3. Effect of different culture medium on the antibacterial activity of strain DNN6. P. vulgaris: *Proteus vulgaris*; B. Subtilis: *Bacillus subtilis*; E. Coli: *Escherichia coli*; C. Perfringens: *Clostridium perfringens*; S. Aureus: *Staphylococcus aureus*

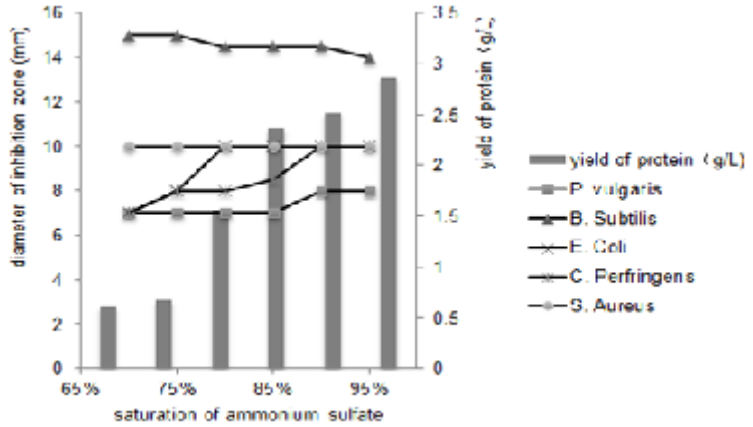

Fig. 4. The effect of ammonium sulfate saturation on yields and antibacterial activity of protein of DNN6. *E. coli: Escherichia coli*; B. Subtilis: *Bacillus subtilis*; P. vulgaris: *Proteus vulgaris*; C. Perfringens: *Clostridium perfringens*; S. Aureus: *Staphylococcus aureus*

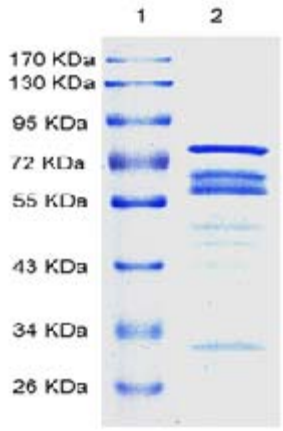

Fig. 5. SDS-PAGE result of the proteins precipitated by 95% saturation ammonium sulfate. Lane 1: Standard molecular weight markers; Lane 2: Protein extracted from strain DNN6

trend with an increased pH value. The time, concentration of sucrose and peptone showed no significant influence on the antibacterial activity compared with the temperature and pH. There appear to be an increased trend with increase of pH, time, and concentration of sucrose when the culture temperature is kept at 25°C. Comprehensive consideration of these results and combined with K value, the optimal culture conditions for antibacterial activity were that the strain DNN6 was fermented in the medium with 50 g/L sucrose and 10 g/L peptone and pH 9 at 25°C for 6 d. The antibacterial activity of the fermented broth illustrated in figure 3 was obviously better than that without optimal culture.

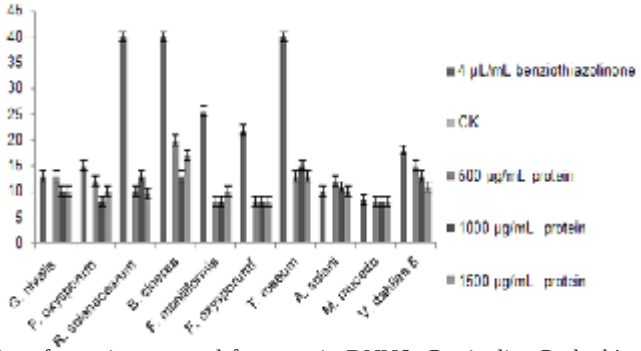

Fig. 6 Antifungal activity of protein extracted from strain DNN6. G. nivalis: *Gerlachia nivalis* (*Ces*, ex *Sacc*.); F. oxysporum: *Fusarium oxysporum* Schlecht; R. solanacearum: *Ralstonia solanacearum* (Burkholderia, Pseudomonas); B. cinerea: *Botrytis cinerea* Pers; F. moniliformis: *Fusarium moniliformis* intermedium Neish & M. Legg; F. oxysporumf: *Fusarium oxysporum* f sp. *Melonis* (muskmelon); T. roseum: *Trichothecium roseum* (Bull.) Link; A. solani: *Alternaria solani* Sorauer; M. mucedo: *Mucor mucedo* (L.) Brefeld; V. dahlia: VD-8 of *Verticillium dahliae*

Extraction of Antimicrobial Proteins

Although the fermentation broth appears obvious antibacterial activity, the active ingredient is indefinable. As the antibacterial proteins or peptides from microorganism have been reported[26-29], the proteins in the fermentation broth of strain DNN6 were extracted and the antibacterial activity of the proteins was detected.

Ammonium sulfate was used for salting out the antibacterial protein. To obtain higher yield and better antibacterial activity protein, 70%, 75%, 80%, 85%, 90% and 95% saturation of ammonium sulfate was used respectively. The results of precipitation and antibacterial activity are iliustrated in Figure 4. The proteins yield increases with increased saturation of ammonium sulfate. The highest yield is 2.866 g/L when the fermentation brooth was added into a 95% saturation of ammonium sulfate. The antibacterial activity of the proteins against to *Proteus vulgaris, Clostridium perfringens and Escherichia coli* increased with increased saturation, while that against *Bacillus subtilis* decreased slightly. The antibacterial activity to *Staphylococcus aureus* showed no difference for all saturation of ammonium sulfate. Considering all these results the 95% saturation was chosen to precipitate the proteins, and the proteins obtained from it was analyzed by SDS-PAGE (Figure 5). Result indicated that there were three protein bands between 55 kD and 95 kD, two protein bands between 43 kD and 55 kD, and one protein band between 26 kD and 34 kD in this crude protein.

Antifungal activity of the crude protein

To exploit the applied potentiality the antifungal activity of the protein of strain DNN6 was detected. Results illustrated in Figure 6 indicated that five of ten different kinds of fungi including *Gerlachia nivalis* (*Ces*, ex *Sacc*.), *Ralstonia solanacearum* (Burkholderia, Pseudomonas), *Botrytis cinerea* Pers, *Trichothecium roseum* (Bull.) Link and *VD-8 of Verticillium dahlia* were strongly inhibited by protein of DNN6, the largest diameter of inhibition zones for these strains was 13, 13, 20, 15 and 15 mm in the above order. The protein of DNN6 also presents antifungal activity to the other five fungi in a way. The results of protein from Bacillus having antifungal activity are consistent with previous reports[30,31]. The protein of strain DNN6 showed a broad antimicrobial spectrum.

CONCLUSION

A novel endophyte *Bacillus licheniformis* strain DNN6 from *Laminaria Japonica* Aresch was isolated and identified. High antibacterial activity was achieved by cultivation in a medium with 50 g/L sucrose, 10 g/L peptone and pH 9 at 25°C for 6 d. The high yield (2.866 g/L) and antibacterial activity of of proteins from strain DNN6 was precipitated by 95% saturation of ammonium sulfate. Moreover antifungal activity detection demonstrated that this protein had a broad antimicrobial spectrum. Therefore, results suggest that the protein from endophyte *Bacillus licheniformis* strain DNN6 is a potential biomaterial for antimicrobial activity.

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