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紫菜腐霉拮抗菌的筛选和鉴定*

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摘要 生物防治广泛用于农作物的病害防治, 该方法在藻类病害防控方面尚未有相关的报道。腐霉(*Pythium* sp.)是引起紫菜(*Neopyropia*)赤腐病(red rot disease)的主要病原, 本研究的目的是筛选和鉴定对紫菜腐霉有拮抗能力的细菌。从养殖藻类及其养殖环境中分离鉴定了 385 株细菌, 通过平板对峙法筛选到 9 株对腐霉有拮抗作用的细菌, 进一步通过含毒介质法筛选到 3 株胞外产物对腐霉具有抑制活性的细菌(P3、P6 和 P19)。3 株拮抗菌对 8 株腐霉均有拮抗活性, 对腐霉生长的抑制率分别为 52.09%~97.95% (P3)、26.81%~78.04% (P6)、10.47%~41.91% (P19)。通过 16S rRNA 鉴定和多位点序列进化分析(16S rRNA-*dnaA*-*dnaN*-*recA*), 将 P3 和 P6 鉴定为杀鱼假交替单胞菌(*Pseudoalteromonas piscicida*), P19 鉴定为解肽假交替单胞菌(*Pseudoalteromonas peptidolytica*)。本研究筛选得到的拮抗菌为下一步建立紫菜赤腐病的生物防治方法奠定了基础。

关键词 腐霉; 拮抗菌; 假交替单胞菌; 生物防治

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紫菜(*Neopyropia*)是一类生长于潮间带的大型经济红藻, 广泛分布于寒带至热带海域, 具有较高的经济价值及生态价值(章守宇等, 2019; 江涛等, 2021)。我国是紫菜生产大国, 紫菜的栽培规模及产量均居世界之首, 年产值约 13 亿美元(http://www.fao.org/fishery/statistics/global-aquaculture production/zh)。然而, 由细菌、卵菌和病毒等引起的病害频繁发生, 给养殖户带来了巨大的经济损失(Arasaki, 1947; Migita, 1969; Saito *et al*, 1972; Kim *et al*, 2016)。其中, 由卵菌病原腐霉(*Pythium* sp.)引起的赤腐病(red rot disease)是紫菜栽培期最主要的病害, 常导致紫菜病烂、空帘而绝收(Arasaki, 1947; Takahashi, 1970; Lee *et al*, 2015)。目前, 生产上经常采用干出、冷藏、酸洗的方法来应对紫菜赤腐病(Fujita *et al*, 1980; Sakaguch *et al*, 2001; Akizuki *et al*, 2007)。将患病紫菜干燥至含水量为 30%~40%并在–20℃冷藏 1~2 周, 可使紫菜腐霉菌丝大量死亡, 但紫菜腐霉的卵孢子对干燥及冷藏具有较好的耐受性, 存活率为 10%~30% (Fujita *et al*, 1980)。用 pH 2.0~5.0 的无机酸或有机酸处理患病紫菜, 在腐霉孢子浓度较低的情况下可有效抑制赤腐病, 但在腐霉孢子浓度较高时处理效果不明显(Sakaguchi *et al*, 2001; Akizuku *et al*, 2007)。此外, 这些物理或化学的

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处理方法还存在一系列问题, 如冷藏设备及场地会大幅度提高栽培成本; 酸性杀菌剂不仅污染栽培海区, 长期使用还会提高腐霉的耐酸性而影响防治效果(Hwang *et al*, 2009)。多年来, 研究人员一直尝试选育或培育紫菜抗病品系, 到目前为止还未得到对赤腐病完全免疫的品系(Polne-Fuller *et al*, 1984; Park *et al*, 2014、2015)。

在陆地农作物生产中利用有益的拮抗微生物或其代谢产物进行病害防控是一种绿色、安全、有效的方法(王丁等, 2018), 这为经济海藻的病害防控提供了新思路。藻类及其生长环境存在多种多样的微生物, 且处在动态变化中, 这些微生物分泌的多种活性物质具有抗菌、抗病毒、抗寄生虫等活性(Singh *et al*, 2014、2015; 阎永伟等, 2022), 是筛选生防微生物的良好来源。本研究的目的是从海藻及其生长环境中筛选和鉴定出对紫菜腐霉具有拮抗作用的细菌, 为建立紫菜赤腐病的生物防治奠定基础。

1　材料与方法

1.1　供试菌株及培养基

紫菜赤腐病病原为腐霉 *Pythium porphyrae* 和 *Pyt. chondricola*。本研究所用的 *Pyt. porphyrae* 菌株(NBRC 30800、NBRC 33126、NBRC 100633 和 NBRC 33253)购自日本生物资源库(NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Japan), *Pyt. chondricola* 菌株(JS151205、PYTHT201801-1、RZ201902 和 LS201903)由本实验室分离自患赤腐病紫菜(邱丽萍, 2018; 何礼娟, 2020)。上述菌株保藏于中国水产科学研究院黄海水产研究所养殖生物病害控制与分子病理学研究室, 在 4℃传代保藏。培养紫菜腐霉的培养基为海水玉米培养基(SCM) (玉米粉 2 g/L、酵母粉 1 g/L、琼脂 15 g/L、盐度为 20 的海水、pH 调为 7.0~7.5), 常规培养温度为 24℃。培养细菌的培养基为 2216E 海水培养基(MA) (蛋白胨 5 g/L、酵母膏 1 g/L、$FePO_4$ 0.01 g/L、琼脂 15 g/L、陈海水), 常规培养温度 28℃, 震荡条件为 150 r/min。

1.2　细菌的分离和纯化

采集紫菜的苗期贝壳丝状体、海区栽培期的叶状体、海带(*Saccharina japonica*)苗及相应的栽培水体进行细菌的分离。采集的样品放入无菌采样袋(海博生物)及水样采集袋(海博生物)带回实验室处理。紫菜贝壳丝状体用灭菌海水清洗表面 3 次, 用无菌刮刀刮取约 1 cm^2 贝壳丝状体; 紫菜叶状体及海带苗用无菌刷子清除表面附着的杂质, 再用灭菌海水清洗 3 次。所得的藻类样品用无菌研磨棒分别匀浆, 用灭菌海水分别将匀浆液进行梯度稀释, 取稀释度为 10^{-2}、10^{-3}、10^{-4} 的稀释液 100 μL 涂布于 MA 上。水样也分别稀释至 10^{-1}、10^{-2} 和 10^{-3}, 取 100 μL 涂布于 MA 上。将所有平板置于 28℃培养 2 d, 挑取形态不同的单菌落进行纯化, –80℃保存在 30%甘油里。

1.3　拮抗菌初筛

用平板对峙法进行拮抗菌的初筛(Farhaoui *et al*, 2022)。用接种环挑取细菌菌株在 MA 上划线, 在 28℃培养活化 2 d 备用。供试紫菜腐霉菌株 NBRC 33253 接种在 SCM 上, 在 24℃培养 7 d 至长满平板后, 用打孔器沿边缘菌丝将紫菜腐霉打成 5 mm 的圆形菌饼, 将其接种在新的 SCM 平板中央, 于 24℃培养 1 d 后, 以紫菜腐霉菌饼为中心画“十”字作为定位线, 挑取细菌菌落在距离紫菜腐霉菌饼中心 2 cm、垂直于定位线画 1 cm 短线, 每平板可划 4 条短线, 即接种 4 株菌株, 对照组不接种细菌。将平板置于 24℃培养, 为高效筛得目标菌株, 本实验选用腐霉最适生长温度 24℃(何礼娟, 2020), 待对照组紫菜腐霉直径长至约为 4 cm 时, 观察接种细菌的周围是否出现抑菌圈。选出对紫菜腐霉有拮抗效果的菌株重复上述实验, 在同一平板上接种相同菌株, 每组 3 个平行, 用游标卡尺量取定位线上紫菜腐霉边缘与细菌之间的距离。对具有拮抗能力的菌株重复上述筛选 2 次。

1.4　拮抗菌复筛

用含毒介质法进行拮抗菌的复筛(何碧珀等, 2019)。将初筛得到的细菌单菌落接种到 100 mL MA 液体培养基中, 在 28℃、150 r/min 条件下震荡培养 72 h, 取 30 mL 菌液于 50 mL 离心管中 5000 r/min 离心 10 min, 取上清液用 0.22 μm 膜过滤获得无菌滤液。将灭菌 SCM 冷却至 40℃左右, 按无菌滤液∶SCM= 1∶5 的比例充分混匀后制备含无菌滤液平板。将紫菜腐霉 NBRC 33253 制成 5 mm 菌饼接种在含拮抗菌无菌滤液的平板中央, 作为实验组; 根据初筛结果选择 1 株无拮抗效果的菌株制备无菌滤液, 用上述方法制备平板, 接种紫菜腐霉菌饼作为阴性对照组; 在不含无菌滤液的 SCM 平板上接种紫菜腐霉菌饼作为空白对照组。每组平板设 3 个平行。将平板置于 24℃培养, 至空白对照组紫菜腐霉长满平板(约 7 d), 用菌落分析仪(Synbiosis Protocol 2, 英国)拍摄平板照片, 然后用显微镜(OLYMPUS BX53, 日本)的自带软件

AJ-VERT 的“任意多边形”工具圈取腐霉生长面积，计算抑菌率。重复上述实验 2 次。

计算公式：抑菌率=$(A_b–A_e)/(A_b–A_i)\times100\%$

式中，A_b 为空白对照组的腐霉面积，A_e 为实验组的腐霉面积，A_i 为菌饼面积。

1.5 拮抗谱的测定

用平板对峙法测定 3 株拮抗菌(P3、P6 和 P19)对 8 株腐霉的拮抗谱及拮抗能力。具体步骤为：挑取单菌落接种到 MA 液体培养基培养 12 h，按 10%的接种量转接至新鲜的 MA 液体培养基继续培养 1~2 h，将细菌培养液 5000 r/min 离心 10 min 去除上清液，用灭菌海水悬浮细菌，重复离心、悬浮步骤 3 次，最后制成 10^8 CFU/mL 的菌悬液备用。将 5 mm 紫菜腐霉菌饼接种在 SCM 中央，1 d 后按 **1.3** 中的定位方法，在距离菌饼 2 cm 处，点种菌悬液 2 μL 作为实验组，点种 2 μL 灭菌海水作为空白对照组。每组设 3 个平行，将所有平板置于 15℃。为筛得具有防治赤腐病潜力的菌株，本实验选用温度为实验室腐霉侵染紫菜温度 15℃，待空白组腐霉长满平板(10~15 d)，计算抑制率，统计方法同 **1.4**。重复上述实验 2 次。

用打孔器将抑菌圈边缘的腐霉菌丝打成 5 mm 直径的菌饼，用乳酸酚棉蓝染液(G1600, Solarbio)对腐霉菌饼进行染色，染色完成后用灭菌海水洗去菌饼表面残余的染液后制片，以空白对照组腐霉边缘菌丝为对照，用显微镜观察抑菌圈边缘菌丝的形态变化。

1.6 拮抗细菌的鉴定

对细菌进行菌落形态观察、革兰氏染色(G1060, Solarbio)。用通用引物 27F/1492R 扩增拮抗菌的 16S rRNA 基因进行细菌鉴定(杨慧超, 2019)。对假交替单胞菌属细菌(*Pseudoalteromonas* sp.)进一步扩增管家基因 *dnaA*、*dnaN*、*recA* 基因序列，进行多位点序列分析(multilocus sequence analysis, MLSA)。根据 GenBank 中假交替单胞菌的 *dnaA*、*dnaN*、*recA* 序列，利用 BioEdit 软件(Alzohairy, 2011)，识别序列中的高保守区域，设计引物。*dnaA* 的引物序列为 PdnaAF (5′-TTTAGCATGTGGGTAAGACC-3′)和 PdnaAR (5′-TCTTGGTCTAGCAACTGAAC-3′)，*dnaN* 为 PdnaNF (5′-TGCAAATAACGATTCCAAGAG-3′)和 PdnaNR (TTTGCATCAGAAAGCGTAAA)，*recA* 为 recA-F/recA-R (Beurmann *et al*, 2017)。利用 MEGA-X 软件将 16S rRNA (1499 bp)、*dnaA* (1146 bp)、*dnaN* (955 bp)和 *recA* (811 bp)基因序列按顺序连接，并进行比对，构建 N-J 系统发育树。

1.7 数据分析

利用 Excel 和 SPSS 软件对实验数据进行统计分析，实验结果取平均值±标准差 ($\overline{x}\pm SD, n=3$)，利用 Duncan 检验法进行多重比较，显著性为 $P<0.05$。

2 结果

2.1 细菌的分离及拮抗菌筛选

从紫菜的贝壳丝状体、叶状体、海带及栽培水体中分离到 385 株细菌用于筛选拮抗菌。用平板对峙法进行初筛时，得到 9 株对腐霉 NBRC 33253 具有拮抗作用的菌株(图 1)。2 次重复实验显示，拮抗菌的抑菌带宽度在 1.65~16.54 mm 之间(表 1)。

用含毒介质法对这 9 株细菌进行复筛时，有 3 株菌株(P3、P6 和 P19)的无菌滤液对腐霉的生长产生了显著的抑制(图 2)。2 次重复实验显示，这 3 株细菌的抑菌率为 20.04%~30.09% (表 1)。根据这些结果，选取 P3、P6 和 P19 进行下一步实验。

2.2 拮抗谱

用平板对峙法检测了 P3、P6 和 P19 对 8 株腐霉的拮抗谱和拮抗能力。2 次重复实验结果显示，15℃条件下，3 株拮抗菌对 8 株腐霉均具有拮抗作用(图 3)，其中，P3 的拮抗能力最强，抑菌率达到(52.09%~97.95%)，其次为 P6 (26.81%~78.04%)和 P19 (10.47%~41.91%) (表 2)。用乳酸棉酚蓝对对峙边缘的腐霉菌丝进行染色观察，结果显示，与对照组的腐霉菌丝相比，与拮抗菌 P3 和 P19 对峙的腐霉菌丝密度变稀疏、着色变浅，而与拮抗菌 P6 对峙的腐霉菌丝无明显变化(图 4)。

2.3 拮抗菌的鉴定

菌株 P3、P6 和 P19 革兰氏染色均为阴性，其中，P6 产黄色色素，P19 产棕色色素，P3 无色素产生。16S rRNA 基因序列分析结果显示，3 株拮抗菌均与假交替单胞菌的相似性最高，P3 和 P6 为 99.93%，P19 为 99.86%。将 P3、P6 和 P19 的 4 个基因串联(16S rRNA-*dnaA*-*dnaN*-*recA*)构建多位点序列的系统进化树，分析结果显示，P3 和 P6 与杀鱼假交替单胞菌(*Pseudoalteromonas piscicida*)聚为一支，P19 与解肽假交替单胞菌(*Pseudoalteromonas peptidolytica*)聚为一支(图 5)。

3 讨论

大型海藻在生长发育过程中会产生多种代谢物质到藻体表面，为微生物定植提供了适宜的基质和营养(Steinberg *et al*, 2002; Staufenberger *et al*, 2008;

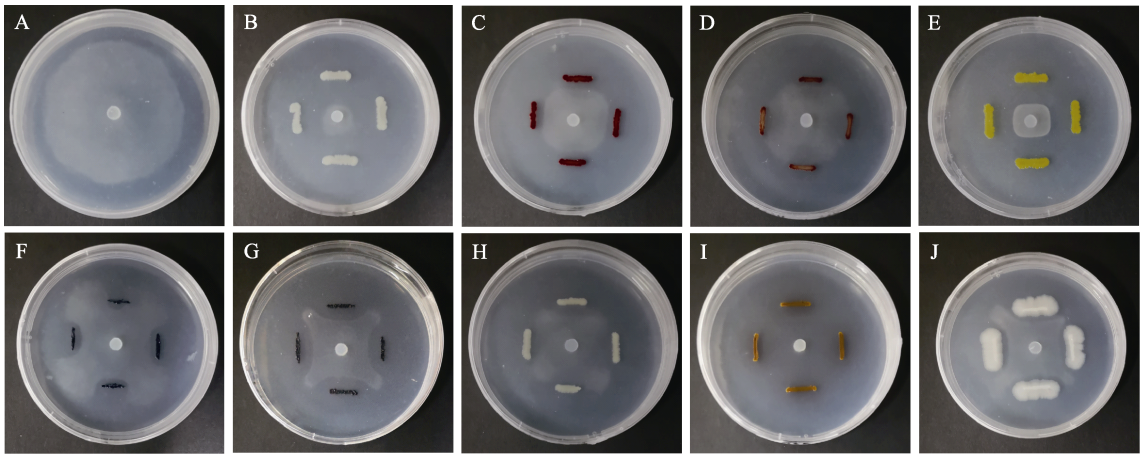

图 1　对峙生长法初筛腐霉拮抗菌

Fig.1　Preliminary screening of *Pyt. porphyrae* antagonistic bacteria by dual culture bioassays

A：空白对照；B~J：P3(B)、P4(C)、P5(D)、P6(E)、P7(F)、P8(G)、P12(H)、P19(I)、B1(J)与腐霉的对峙生长

A: Blank control; B~J: Antagonistic growth of P3(B), P4(C), P5(D), P6(E), P7(F), P8(G), P12(H), P19(I), and B1(J) against *Pyt. porphyrae*

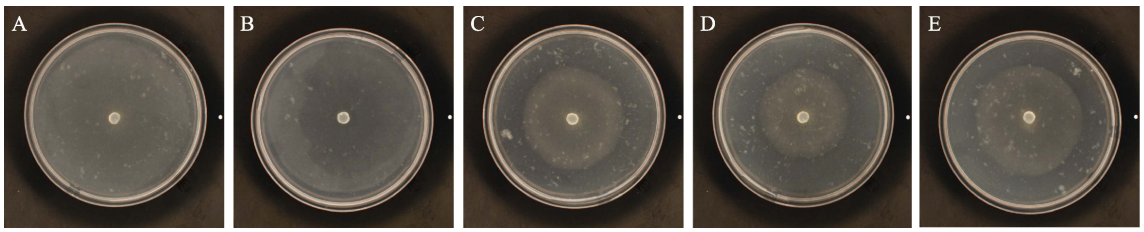

图 2　含毒介质法复筛拮抗菌

Fig.2　Secondary screening of antagonistic bacteria by toxic media method

A：空白对照；B：阴性对照组；C~E：腐霉在含有 P3(C)、P6(D)、P19(E)无菌滤液的培养基上生长

A: Blank control; B: Negative control;

C~E: *Pyt. porphyrae* grew on the medium containing extracellular products from P3(C), P6(D) and P19(E)

表 1　拮抗菌的筛选

Tab.1　Screening of antagonistic bacteria

组别 Group	初筛/(抑菌带/mm) Preliminary screening/(Bacteriostatic zone/mm)		复筛/(抑制率/%) Secondary screening/(inhibition rate/%)	
	第 1 次 Test 1	第 2 次 Test 2	第 1 次 Test 1	第 2 次 Test 2
P3	11.79±0.90^{f}	12.25±1.60^{f}	21.51±2.59^{a}	20.04±1.56^{a}
P4	3.66±0.31^{c}	4.19±0.09^{c}	0	0
P5	1.70±0.39^{a}	1.65±0.37^{a}	0	0
P6	10.58±0.20^{e}	10.95±0.46^{e}	23.12±2.45^{a}	21.56±2.53^{a}
P7	2.87±0.16^{b}	3.11±0.22^{b}	0	0
P8	6.19±0.19^{d}	6.60±0.36^{d}	0	0
P12	2.58±0.67^{b}	2.89±0.42^{b}	0	0
P19	16.37±0.35^{g}	16.54±0.56^{g}	30.09±4.66^{b}	27.66±2.27^{b}
B1	2.80±0.33^{b}	2.79±0.37^{b}	0	0
对照 Control[1]	0	0	/	/
空白对照 Blank control[2]	/	/	0	0
阴性对照 Negative control[3]	/	/	0	0

注：1. 初筛实验中仅接种腐霉的平板；2. 复筛实验中，在不含菌液的 SCM 平板上接种腐霉；3. 复筛实验中，在含有无拮抗作用细菌菌液的 SCM 平板上接种腐霉。同列不同字母上标表示数据存在显著差异，相同字母表示数据无显著差异。

Note: 1. The control group in the primary screening experiment; 2. The control group in the secondary screening experiment; 3. Negative control group in the secondary screening experiment. Different superscripts indicate significant differences in the same column, while the same superscripts indicate no significant differences.

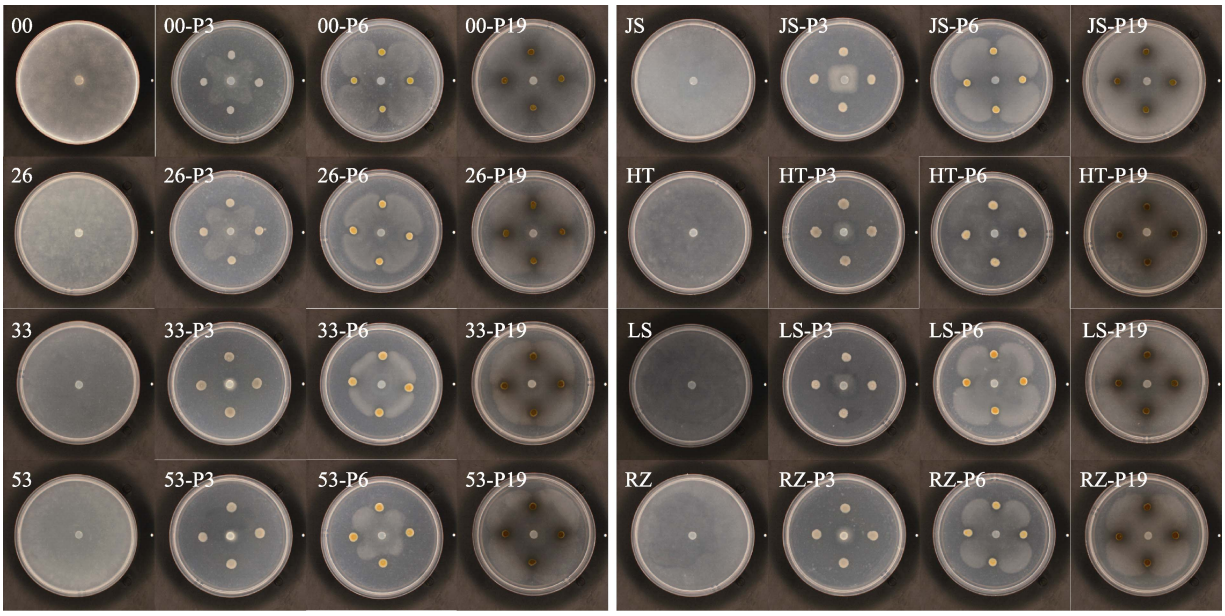

图 3 8 株腐霉与 3 株拮抗菌的对峙生长图

Fig.3 Confrontation growth between *Pythium* and antagonistic bacteria

00、26、33、53、JS、HT、LS、RZ 分别为腐霉 NBRC 30800、NBRC 33126、NBRC 100633、NBRC 33253、JS151205、PYTHT201801-1、LS201903、RZ201902；00-P3、26-P3、33-P3、53-P3、JS-P3、HT-P3、LS-P3、RZ-P3 分别为 P3 与腐霉 NBRC 30800、NBRC 33126、NBRC 100633、NBRC 33253、JS151205、PYTHT201801-1、LS201903、RZ201902 的对峙生长；00-P6、26-P6、33-P6、53-P6、JS-P6、HT-P6、LS-P6、RZ-P6 分别为 P6 与腐霉 NBRC 30800、NBRC 33126、NBRC 100633、NBRC 33253、JS151205、PYTHT201801-1、LS201903、RZ201902 的对峙生长；00-P19、26-P19、33-P19、53-P19、JS-P19、HT-P19、LS-P19、RZ-P19 分别为 P19 与腐霉 NBRC 30800、NBRC 33126、NBRC 100633、NBRC 33253、JS151205、PYTHT201801-1、LS201903、RZ201902 的对峙生长。

00, 26, 33, 53, JS, HT, LS, RZ represent *Pythium* NBRC 30800, NBRC 33126, NBRC 100633, NBRC 33253, JS151205, PYTHT201801-1, LS201903, RZ201902, respectively; 00-P3, 26-P3, 33-P3, 53-P3, JS-P3, HT-P3, LS-P3, RZ-P3 represent antagonistic growth of P3 against *Pythium* NBRC 30800, NBRC 33126, NBRC 100633, NBRC 33253, JS151205, PYTHT201801-1, LS201903, RZ201902, respectively; 00-P6, 26-P6, 33-P6, 53-P6, JS-P6, HT-P6, LS-P6, RZ-P6 represent antagonistic growth of P6 against *Pythium* NBRC 30800, NBRC 33126, NBRC 100633, NBRC 33253, JS151205, PYTHT201801-1, LS201903, RZ201902, respectively; 00-P19, 26-P19, 33-P19, 53-P19, JS-P19, HT-P19, LS-P19, RZ-P19 represent antagonistic growth of P19 against *Pythium* NBRC 30800, NBRC 33126, NBRC 100633, NBRC 33253, JS151205, PYTHT201801-1, LS201903, RZ201902, respectively.

表 2 拮抗菌对腐霉生长的抑菌率

Tab.2 Growth inhibition rate of antagonistic bacteria against *Pythium* strains

编号 No.	抑制率 Inhibition rate /%					
	第 1 次 Test 1			第 2 次 Test 2		
	P3	P6	P19	P3	P6	P19
NBRC 30800(00)	83.57±1.78^{a}	49.66±7.77^{b}	26.74±2.59^{c}	57.58±2.45^{a}	45.37±2.29^{b}	14.38±7.95^{c}
NBRC 33126(26)	86.35±6.39^{a}	47.01±4.10^{b}	35.98±3.15^{c}	52.09±4.28^{a}	39.63±3.35^{b}	31.67±1.89^{c}
NBRC 100633(33)	97.41±0.27^{a}	65.94±0.38^{b}	41.91±4.72^{c}	97.95±0.75^{a}	53.67±7.26^{b}	17.90±2.13^{c}
NBRC 33253(53)	97.92±0.13^{a}	78.04±0.13^{b}	33.16±3.20^{c}	96.31±0.21^{a}	66.23±6.08^{b}	23.03±3.44^{c}
JS151205(JS)	92.23±0.31^{a}	35.84±3.40^{b}	18.67±0.24^{c}	86.27±0.82^{a}	26.81±4.14^{b}	10.47±1.42^{c}
PYTHT201801-1(HT)	95.89±0.54^{a}	30.71±2.28^{b}	19.12±5.30^{c}	94.89±0.34^{a}	45.55±4.17^{b}	20.26±2.93^{c}
LS201903(LS)	95.47±0.56^{a}	48.36±4.32^{b}	16.37±2.56^{c}	96.95±0.57^{a}	35.80±6.32^{b}	21.11±0.89^{c}
RZ201902(RZ)	96.82±0.49^{a}	59.69±0.53^{b}	40.29±3.04^{c}	96.82±0.72^{a}	57.03±5.56^{b}	35.50±7.35^{c}

注：将 3 株拮抗菌对同一腐霉的生长抑制率进行差异显著性分析，同行不同字母上标表示数据存在显著差异，相同字母表示数据无显著差异。

Note: The growth inhibition rates of three antagonistic bacteria against the same *Pythium* strain were analyzed. Different superscripts indicate significant differences in the same row, while the same superscripts indicate no significant differences.

Singh *et al*, 2013)。附着在海藻表面的微生物群落可产生多种活性物质，这些物质不仅影响海藻的正常形态及生长发育，还可保护宿主免受有害生物的侵害。因此，大型海藻的附着微生物成为生物和医药领域的优选目标(Singh *et al*, 2014、2015)。本研究从紫菜和海带及其栽培水体分离细菌，通过平板对峙法筛选出 9 株具有抑制腐霉生长活性的细菌，并通过含毒介质法检测出 3 株细菌(P3、P6 和 P19)的胞外产物能够抑制腐霉的生长，表明这 3 株细菌通过拮抗作用抑制了腐霉的生长。其他 6 株细菌的胞外产物未检测到抑制腐霉生长的情况，说明这 6 株细菌通过竞争作用抑制了腐霉的生长。

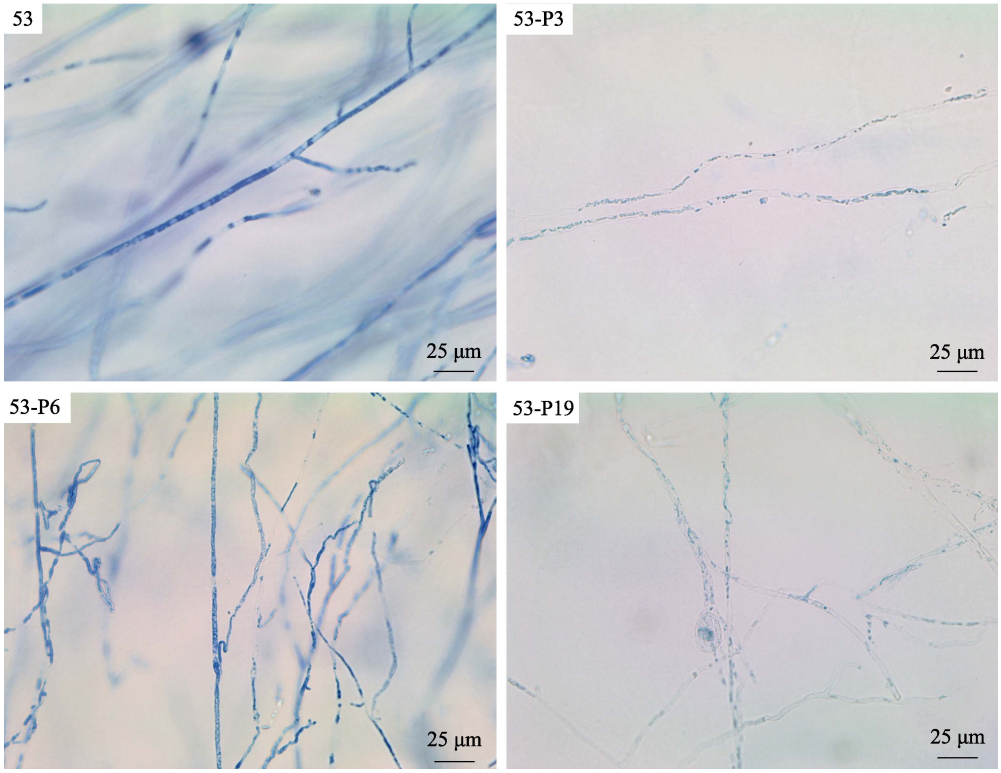

图 4　拮抗菌和腐霉对峙区域的腐霉菌丝显微观察(乳棉酚蓝染色)

Fig.4　Microscopic observation of the *Pythium* mycelia growing in the confrontation area between *Pythium* and antagonistic bacteria (lactophenol cotton blue staining)

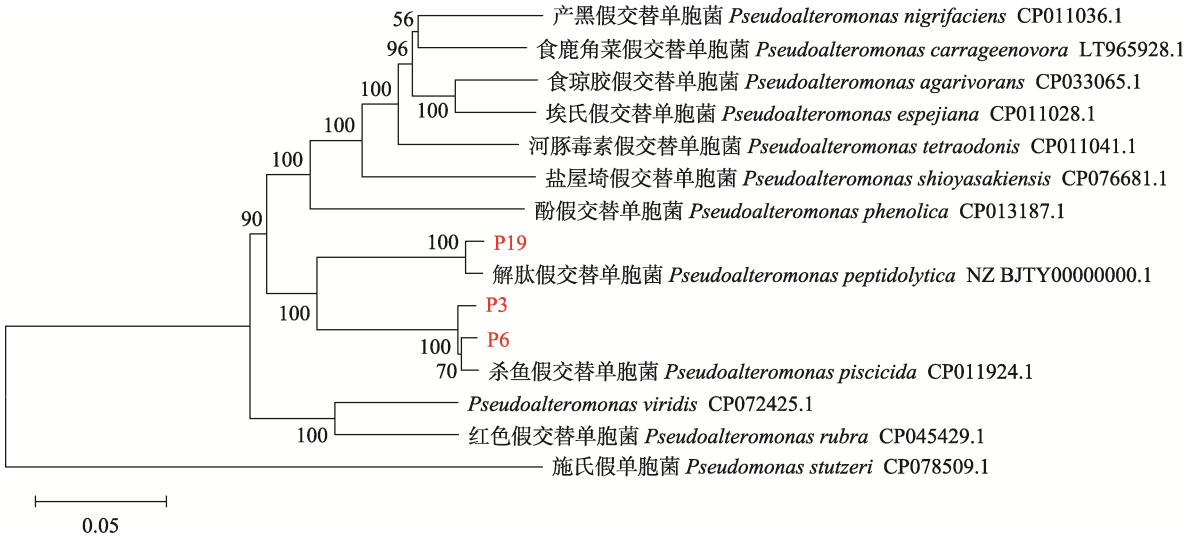

图 5　基于拮抗菌 P3、P6 和 P19 的 16S rRNA-*dnaA*-*dnaN*-*recA* 构建的 N-J 系统发育树

Fig.5　N-J phylogenetic tree based on 16S rRNA-*dnaA*-*dnaN*-*recA* of P3, P6, and P19

通过 16S rRNA 鉴定及多位点序列分析，将 P3 和 P6 鉴定为杀鱼假交替单胞菌，将 P19 鉴定为解肽假交替单胞菌。多个研究表明，从海洋环境分离的假交替单胞菌能分泌具有抗菌、防污、杀藻等作用的活性物质，包括抗生素、胞外酶类物质和胞外毒素等(Holmstrom *et al*, 1999; Egan *et al*, 2001; Bowman, 2007; 孙星等, 2018; 练小军等, 2020)。大多数的研究报道了抗细菌活性物质的分离和鉴定，而对抗真菌、抗卵菌的活性物质的报道不多。Moree 等(2014)报道了从健康珊瑚分离的一株假交替单胞菌产生的一种酰胺聚酮具有抗真菌活性；Franks 等(2005)报道了一株 *P. tunicata* 产生的黄色色素具有抗真菌活性，并鉴定为生物碱。分离自海洋环境的一些杀鱼假交替单胞菌菌株被证实能够有效拮抗多种病原菌，包括创伤弧菌(*Vibrio vulnificus*)、副溶血弧菌(*V. parahaemolyticus*)、美人鱼发光杆菌(*Photobacterium damselae*)、金黄色葡萄球菌(*Staphylococcus aureus*)、白色念珠菌(*Candida albicans*)及枯草芽孢杆菌(*Bacillus subtilis*)等(Richards *et al*, 2017; Eliseikina *et al*, 2021)；一些解肽假交替单胞菌具有淀粉酶、几丁质酶、木质纤维素酶活性(Venkateswaran *et al*, 2000; Johnson *et al*, 2021)。但这些分离株产生的抑菌物质尚不清楚。在本研究中，拮抗菌 P3 和 P19 与腐霉相互作用区域的腐霉菌丝出现乳酸棉酚蓝着色浅的情况，而与 P6 相互作用区域的菌丝无明显变化，说明 3 株细菌可能通过不同方式抑制腐霉生长。当阳性菌株溶解或死亡时，乳酸酚棉蓝染色常呈阴性，说明 P3 和 P19 分泌的活性物质有可能降解了腐霉菌丝或影响菌丝活性，导致着色变浅。

在水产养殖中，假交替单胞菌作为益生菌在防治养殖动物的病害中显示了良好的效果(Fjellheim *et al*, 2010; Goulden *et al*, 2012; Offret *et al*, 2019)。本研究筛选得到的拮抗菌 P3、P6 和 P19，对引起紫菜赤腐病的致病性腐霉展示了显著的拮抗能力，显示了其作为益生菌用于防治紫菜赤腐病的生防潜力。在后续的研究中，我们将开展拮抗菌的安全性评价及其在预防和治疗紫菜赤腐病中的生防效果和应用，在此基础上探究拮抗菌的活性物质的鉴定及其生防机制。

参 考 文 献

AKIZUKI A, TABATA M, KAWAMURA Y. Disinfectant effects of lactic acid on *Pythium porphyrae* as acid treatment agent. Aquaculture Science, 2007, 55(3): 325–330

ALZOHAIRY A M. BioEdit: An important software for molecular biology. GERF Bulletin of Biosciences, 2011, 2(1): 60–61

ARASAKI S. Studies on the rot of *Porohyra tenera* by *Pythium*. Nippon Suisan Gakkaishi, 1947, 13: 74–90

BEURMANN S, USHIJIMA B, VIDEAU P, *et al*. *Pseudoalteromonas piratica* strain OCN003 is a coral pathogen that causes a switch from chronic to acute *Montipora* white syndrome in *Montipora capitata*. PLoS One, 2017, 12(11): e0188319

BOWMAN J P. Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*. Marine Drugs, 2007, 5(4): 220–241

EGAN S, HOLMSTRÖM C, KJELLEBERG S. *Pseudoalteromonas ulvae* sp. nov., a bacterium with antifouling activities isolated from the surface of a marine alga. International Journal of Systematic and Evolutionary Microbiology, 2001, 51(4): 1499–1504

ELISEIKINA M G, BELENEVA I A, KUKHLEVSKY A D, *et al*. Identification and analysis of the biological activity of the new strain of *Pseudoalteromonas piscicida* isolated from the hemal fluid of the bivalve *Modiolus kurilensis* (F. R. Bernard, 1983). Archives of Microbiology, 2021, 203(7): 4461–4473

FARHAOUI A, ADADI A, TAHIRI A, *et al*. Biocontrol potential of plant growth-promoting rhizobacteria (PGPR) against *Sclerotiorum rolfsii* diseases on sugar beet (*Beta vulgaris* L.). Physiological and Molecular Plant Pathology, 2022, 119: 101829

FJELLHEIM A J, KLINKENBERG G, SKJERMO J, *et al*. Selection of candidate probionts by two different screening strategies from Atlantic cod (*Gadus morhua* L.) larvae. Veterinary Microbiology, 2010, 144(1/2): 153–159

FRANKS A, HAYWOOD P, HOLMSTRÖM C, *et al*. Isolation and structure elucidation of a novel yellow pigment from the marine bacterium *Pseudoalteromonas tunicata*. Molecules, 2005, 10(10): 1286–1291

FUJITA Y, MIGITA S. Death of parasitic *Pythium porphyrae* by drying and freeze-preservation of red rot-infected thalli of *Porphyra yezoensis*. Bulletin of the Faculty of Fisheries Nagasaki University, 1980, 49: 11–16

GOULDEN E F, HALL M R, PEREG L L, *et al*. Identification of an antagonistic probiotic combination protecting ornate spiny lobster (*Panulirus ornatus*) larvae against *Vibrio owensii* infection. PLoS One, 2012, 7(7): e39667

HE B P, ZHAO H, LIU H Y, *et al*. Inhibitory effects, growth-promotion evaluation and identification of two biocontrol bacteria against fungal diseases of sesame. Journal of Henan Agricultural Sciences, 2019, 48(11): 92–98 [何碧珀, 赵辉, 刘红彦, 等. 2 株芝麻真菌病害生防菌的抑菌特征、促生评价及鉴定. 河南农业科学, 2019, 48(11): 92–98]

HE L J. Isolation, identification and detection of two pathogenic oomycetes of *Pyropia*. Master´s Thesis of Ocean University

of China, 2020 [何礼娟. 紫菜两种病原性卵菌的分离鉴定及检测. 中国海洋大学硕士研究生学位论文, 2020]

HOLMSTRÖM C, KJELLEBERG S. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiology Ecology, 1999, 30(4): 285–293

HWANG E K, CHAN S P, KAKINUMA M. Physicochemical responses of *Pythium porphyrae* (Oomycota), the causative organism of red rot disease in *Porphyra* to acidification. Aquaculture Research, 2009, 40(15): 1777–1784

JIANG T, FEI F, FANG T. Research progress on the bioactive components of *Porphyra* and its application. Food Research and Development, 2021, 42(21): 162–167 [江涛, 费帆, 方婷. 紫菜生物活性成分及其应用研究进展. 食品研究与开发, 2021, 42(21): 162–167]

JOHNSON J, CHOI K Y. Enzymatic utilization of oil and lignocellulosic biomass using halophilic marine bacteria *Micrococcus luteus* and *Pseudoalteromonas peptidolytica*. 3 Biotech, 2021, 11(7): 360

KIM G H, KLOCHKOVA T A, LEE D J, *et al*. Chloroplast virus causes green-spot disease in cultivated *Pyropia* of Korea. Algal Research, 2016, 17: 293–299

LEE S J, MI S H, PARK M A, *et al*. Molecular identification of the algal pathogen *Pythium chondricola* (Oomycetes) from *Pyropia yezoensis* (Rhodophyta) using ITS and cox1 markers. Algae, 2015, 30(3): 217–222

LIAN X J, ZHU K L, ZHANG Q Q, *et al*. Effects of probiotics-supplemented diets on the growth and survival of *Litopenaeus vannamei* carrying multiple pathogens. Progress in Fishery Sciences, 2020, 41(2): 121–130 [练小军, 朱开玲, 张庆起, 等. 饲料添加益生菌对多病原阳性的凡纳滨对虾生长与存活的影响. 渔业科学进展, 2020, 41(2): 121–130]

MIGITA S. *Olpidiopsis* disease of culture *Porphyra*. Bulletin of the Faculty of Fisheries Nagasaki University, 1969, 28: 131–145

MOREE W J, MCCONNELL O J, NGUYEN D D, *et al*. Microbiota of healthy corals are active against fungi in a light-dependent manner. ACS Chemical Biology, 2014, 9(10): 2300–2308

OFFRET C, ROCHARD V, LAGUERRE H, *et al*. Protective efficacy of a *Pseudoalteromonas* strain in *European Abalone*, *Haliotis tuberculata*, infected with *Vibrio harveyi* ORM4. Probiotics and Antimicrobial Proteins, 2019, 11: 239–247

PARK C S, HWANG E K. Biochemical characterization of *Pyropia yezoensis*-AP1 strain accompanies the resistance reaction to the red rot disease pathogen, *Pythium porphyrae*. Journal of Applied Phycology, 2015, 27(5): 2149–2156

PARK C S, HWANG E K. Isolation and evaluation of a strain of *Pyropia yezoensis* (Bangiales, Rhodophyta) resistant to red rot disease. Journal of Applied Phycology, 2014, 26(2): 811–817

POLNE-FULLER M, GIBOR A. Developmental studies in *Porphyra*. I. blade differentiation in *Porphyra* perforate as expressed by morphology enzymatic digestion, and protoplast regeneration. Journal of Phycology, 1984, 20(4): 609–616

QIU L P. Isolation, identification and characterization of *Pythium porphyrae* JS151205. Master´s Thesis of Ocean University of China, 2018 [邱丽萍. 紫菜腐霉 JS151205 的分离鉴定及生长特性研究. 中国海洋大学硕士研究生学位论文, 2018]

RICHARDS G P, WATSON M A, NEEDLEMAN D S, *et al*. Mechanisms for *Pseudoalteromonas piscicida*-induced killing of vibrios and other bacterial pathogens. Applied and Environmental Microbiology, 2017, 83(11): e00175–17

SAITO Y, MATSUSATO T, YOSHIKAWA K. On the symptoms of "green spot" and "crape" in Nori (*Porphyra*) culture. Bulletin of Nansei National Fisheries Research Institute, 1972, 5: 1–9

SAKAGUCHI K, PARK C S, KAKINUMA M, *et al*. Effects of varying temperature, salinity, and acidity in the treatment of *Porphyra* infected by red rot disease. Japanese Society for Aquaculture Science, 2001, 49(1): 77–83

SINGH R P, KUMARI P, REDDY C R K. Antimicrobial compounds from seaweeds-associated bacteria and fungi. Applied Microbiology and Biotechnology, 2015, 99(4): 1571–1586

SINGH R P, MAHENDRA K S, MISHRA A, *et al*. Bacterial extracellular polymeric substances and their effect on settlement of zoospore of *Ulva fasciata*. Colloids and Surfaces B: Biointerfaces, 2013, 103: 223–230

SINGH R P, REDDY C R K. Seaweed-microbial interactions: Key functions of seaweed-associated bacteria. FEMS Microbiology Ecology, 2014, 88(2): 213–230

STAUFENBERGER T, THIEL V, WIESE J, *et al*. Phylogenetic analysis of bacteria associated with *Laminaria saccharina*. FEMS Microbiology Ecology, 2008, 64(1): 65–77

STEINBERG P D, DE NYS R, KJELLEBERG S. Chemical cues for surface colonization. Journal of Chemical Ecology, 2002, 28(10): 1935–1951

SUN X, XIANG Y Y, BEN Z Y, *et al*. Optimization of antimicrobial culture condition of *Pseudoalteromonas*.SW-1. Chinese Agricultural Science Bulletin, 2018, 34(33): 96–100 [孙星, 向玉勇, 贲宗友, 等. 假交替单胞菌 *Pseudoalteromonas*. SW-1 抑菌培养条件的优化. 中国农学通报, 2018, 34(33): 96–100]

TAKAHASHI M. Identification of genus *Pythium*. Plant Protection Science, 1970, 24(8): 339–346

VENKATESWARAN K, DOHMOTO N. *Pseudoalteromonas peptidolytica* sp. nov., a novel marine mussel-thread-degrading bacterium isolated from the Sea of Japan. International Journal of Systematic and Evolutionary Microbiology, 2000, 50(2): 565–574

WANG D, YANG X, LIU C L, *et al*. Research progress on biological pesticide. Journal of Green Science and

Technology, 2018(24): 179–181 [王丁, 杨雪, 刘春雷, 等. 生物农药研究进展. 绿色科技, 2018(24): 179–181]

YAN Y W, YANG H C, MO Z L, *et al*. Epiphytic microbial communities associated with *Neopyropia yezoensis* with *Olpidiopsis* disease. Progress in Fishery Sciences, 2022, 43(3): 165–175 [阎永伟, 杨慧超, 莫照兰, 等. 患拟油壶菌病条斑紫菜表面附生菌群分析. 渔业科学进展, 2022, 43(3): 165–175]

YANG H C. Diseases investigation of *Pyropia yezoensis* and development of PCR detection method for *Pyropia yezoensis* green spot disease. Master´s Thesis of Shanghai Ocean University, 2019 [杨慧超. 条斑紫菜(*Pyropia yezoensis*)的病害调查及其绿斑病病原的 PCR 检测. 上海海洋大学硕士研究生学位论文, 2019]

ZHANG S Y, LIU S R, ZHOU X J, *et al*. Ecological function of seaweed-formed habitat and discussion of its application to sea ranching. Journal of Fisheries of China, 2019, 43(9): 2004–2014 [章守宇, 刘书荣, 周曦杰, 等. 大型海藻生境的生态功能及其在海洋牧场应用中的探讨. 水产学报, 2019, 43(9): 2004–2014]

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Screening and Identification of Antagonistic Bacteria Against *Pythium* Causing Red Rot Disease in *Neopyropia*

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Abstract China is the largest producer of *Neopyropia yezoensis*, ranking first in the world for cultivation area and yield production. In *N. yezoensis* production, diseases occur frequently every year due to increased farming density, environmental deterioration, and germplasm degeneration, resulting in serious economic losses to farmers. Red rot disease is caused by *Pythium* sp. and is one of the most common diseases during *N. yezoensis* farming, leading to empty nets and harvest loss. Air-dry, cold storage, and acid wash are common methods to counteract red rot disease in *N. yezoensis* farming. These physical or chemical disinfection methods, however, are not completely effective, and some have serious consequences. For example, refrigeration equipment and space will greatly increase costs, and acid wash treatments can cause environmental pollution. Although research has attempted to select or cultivate disease-resistant strains of laver, there remains no laver strain completely immune to red rot disease. Biocontrol is an effective method that is widely used in disease control of land crops. Biocontrol is potentially an environment-friendly and effective control method for macroalgal diseases. However, limited information exists on biocontrol in macroalgal diseases. During the growth and development of macroalgae, a variety of metabolites are produced on their surfaces, which provide suitable substrates for microbial colonization. The microbial community attached to the surface of algae is highly diverse and can produce many kinds of biologically active compounds. These compounds not only play a major role in normal morphology, growth, and development of algae, but also have antibacterial, antiviral, antiparasitic, and other activities to protect the host from harmful organisms. Therefore, the epiphytic microorganisms of algae provide good sources of microorganisms for biological screening. This study aimed to screen and identify bacteria with antagonistic ability towards *Pythium* sp.. A total of 385 bacterial strains, isolated from farming algae and their culturing environments, were screened. In the first round of screening, the plate confrontation method was used and repeated twice and confirmed that nine

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strains had antagonistic effects on the growth of *Pythium* sp.. The diameter of the bacteriostatic zone was approximately 1.65–16.54 mm. In the second round of screening, three strains (assigned as P3, P6, and P19) were further investigated using the toxic medium method for inhibitory activities in their extracellular products. Repeated experiments showed that the bacteriostatic rate was approximately 20.04%–30.09%. The antibacterial spectrum was determined by the plate confrontation method. Strains P3, P6, and P19 all had antagonistic effects on the eight tested strains of *Pythium* preserved in our laboratory. The inhibition rates reached 52.09%–97.95% for P3, 26.81%–78.04% for P6, 10.47%–41.91% for P19, respectively. The *Pythium* hyphae on the confrontation edge were further investigated by lactic acid phenol cotton blue staining. When compared with *Pythium* hyphae in a control group, the density and color of *Pythium* hyphae against strains P3 and P19 became sparse and lighter. There were no significant changes in *Pythium* hyphae against strain P6. Strains P3 and P6 were identified as *Pseudoalteromonas piscicida*, and P19 as *P. peptidolytica*, based on 16S rRNA gene identification and multilocus sequences analysis of 16S rRNA-*dnaA*-*dnaN*-*recA*. The bacterial strains of P3, P6, and P19 had significant antagonistic capabilities against the pathogenic *Pythium* strains. This indicates they are potential biocontrol probiotics for the control of red rot disease in *N. yezoensis*. The present study provides the foundation for research on the evaluation and application of antagonistic bacterial strains in the biocontrol of red rot disease of *N. yezoensis*.

Key words *Pythium*; Antagonistic bacteria; *Pseudoalteromonas*; Biocontrol