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ORIGINAL PAPER

Production of enzymes and antimicrobial compounds by halophilic Antarctic *Nocardioides* sp. grown on different carbon sources

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Abstract This study demonstrated the potential of microbial isolates from Antarctic soils to produce hydrolytic enzymes by using specific substrates. The results revealed potential of the strains to produce a broad spectrum of hydrolytic enzymes. Strain A-1 isolated from soil samples in Casey Station, Wilkes Land, was identified as *Nocardioides* sp. on the basis of morphological, biochemical, physiological observations and also chemotaxonomy analysis. Enzymatic and antimicrobial activities of the cell-free supernatants were explored after growth of strain A-1 in mineral salts medium supplemented with different carbon sources. It was found that the carbon sources favored the production of a broad spectrum of enzymes as well as compounds with antimicrobial activity against Gram-positive and Gram-negative bacteria, especially *Staphylococcus aureus* and *Xanthomonas oryzae*. Preliminary analysis showed that the compounds with antimicrobial activity produced by the strain A-1 are mainly glycolipids and/or lipopeptides depending on the used carbon source. The results revealed a great potential of the Antarctic *Nocardioides* sp. strain A-1 for biotechnological, biopharmaceutical and biocontrol applications as a source of industrially important enzymes and antimicrobial/antifungal compounds.

Keywords Antarctic soils · Actinomycetes · *Nocardioides* · Psychrophiles · Hydrolytic enzymes · Antimicrobial activity

V. Gesheva · E. Vasileva-Tonkova (✉)
Bulgarian Academy of Sciences, The Stephan Angeloff Institute of Microbiology, Acad. G. Bonchev Str., Bl. 26, 1113 Sofia, Bulgaria
e-mail: evaston@yahoo.com

Introduction

Psychrophilic organisms, in particular bacteria, yeasts, unicellular algae and fungi are able to grow efficiently at sub-zero temperatures and have successfully colonized polar and alpine regions (Gounot and Russell 1999). In Antarctica, microorganisms have been detected in all habitats such as lakes, ponds, rivers, streams, rocks and soils. The habitats differ one from another with respect to nutrients, range of temperature, water activity and other physical–chemical parameters, and the microbial flora varies from one habitat to another. Prokaryotes dominate in many Antarctic ecosystems and play a major role in food chains, biogeochemical cycles and mineralization of pollutants (Nichols et al. 1999).

Cold-active microorganisms have developed various adaptations enabling them to compensate for the deleterious effect of low temperatures and to grow in the extreme environments (Russell 1998; Rampelotto 2010). These challenges include: reduced enzyme activity; decreased membrane fluidity; altered transport of nutrients and waste products; decreased rates of transcription, translation and cell division; protein cold-denaturation; inappropriate protein folding, and intracellular ice formation (D'Amico et al. 2006).

Psychrophilic microorganisms mainly bacteria and their enzymes have assumed considerable importance due to biotechnological applications. The catalytic properties of cold-active enzymes as well as their high specific activity at low and moderate temperatures can be extremely useful in different biotechnological fields, for example, in the detergent and food industries, for the production of fine chemicals and in bioremediation processes (Gerday et al. 2000; Huston 2008; Cavicchioli et al. 2011). Cold-adapted enzymes play a crucial ecological role in recycling the

organic matter in Antarctic environments. Permanent cold climate requires that extracellular enzymes act efficiently at low temperatures, and on the other hand, the low concentration of the particulate organic matter requires efficient extracellular enzymes to achieve an adequate uptake of nutrients. In this regard, selection of samples, techniques for isolation of bacterial strains and screening for enzymes constitutes an important aspect of studies on cold-active microorganisms. Furthermore, these microorganisms appear to be inexhaustible source of new bioactive metabolites with antibiotic effect against pathogenic microbes, and continuous efforts are being made in the research on microbial secondary metabolites with potential applications (Giudice et al. 2007).

In our previous work, seventeen microbial strains were isolated from soil samples of three regions of Antarctica and investigated for their ability to grow on hydrocarbons (Vasileva-Tonkova and Gesheva 2004). Some of the isolates were psychrophilic actinomycetes able to produce enzymes and glycolipid biosurfactants (Vasileva-Tonkova and Gesheva 2004; Gesheva 2009a, 2010). In the present work, the potential capability of the Antarctic isolates to produce hydrolytic enzymes was investigated aiming to find sources of industrially relevant enzymes. Isolate marked as A-1 was selected, and the effect of different carbon sources on the production of hydrolytic enzymes and antimicrobial/antifungal compounds by the strain A-1 was studied.

Materials and methods

Enzymatic activities assay

Agar well diffusion assay was used for testing the enzymatic activities of bacterial cultures and cell-free supernatants of strain A-1. The proteolytic, amylolytic, lipolytic, and ribonuclease (RNase) activities were assayed by using skim-milk (30% v/v), gelatin (1% w/v), soluble starch (0.2% w/v), Tween 80 (1% v/v), and yeast RNA (0.5% w/v) as substrates. Cellulase activity was tested as described by McSweeney et al. (2001) with 2% ball milled cellulose (Whatman) as substrate. A little amount of bacterial cultures, 24-h grown on meat extract-peptone agar (MPA) was shaded through a needle on the test media. In another set of Petri plates, wells with a diameter of 6 mm were cut in the agar using a sterile cork-borer, then 0.1 ml of cell-free supernatants of strain A-1 was added into the wells and allowed to diffuse at room temperature for 2 h. The plates were then incubated in the upright position at 30°C in the dark for 24–48 h for proteolytic, amylolytic and RNase activities, and for 48–96 h for lipolytic and cellulase activity. Three replicates were carried out for each sample.

Taxonomic characterization of strain A-1

Strain A-1 was isolated from Casey Station, Budd Coast, Wilkes Land (Vasileva-Tonkova and Gesheva 2004). Mmorphological, biochemical, physiological features, utilization of carbon sources, enzyme tests and also assays of the cell sugars and amino acids in whole-cell hydrolysate were studied according to Vasileva-Tonkova and Gesheva (2005). Antibiotic formation was observed on MPA after 4–6 days of cultivation at 25°C. Antibiotic activity was determined by agar diffusion method against the following test cultures: *Bacillus subtilis* ATCC 6633, *Sarcina lutea*, *Staphylococcus aureus* 605P, *Escherihia coli*, *Candida tropicalis*, and *Saccaharomyces cerevisiae*.

Growth of strain A-1 on different carbon sources

Strain A-1 was cultivated in 200-ml Erlenmeyer flasks containing mineral salts medium (MSM) with composition as described earlier (Vasileva-Tonkova and Gesheva 2004). The medium was supplemented with 2% of different carbon sources; phenanthrene was used in concentration 10 g l^{-1} (Table 4). A control flask without any carbon source was also prepared. The pH value was adjusted to 7.0 ± 0.2 and the media were autoclaved at 121 °C for 15 min. Culture after 24 h of growth in meat extract-peptone broth (MPB) was used as an inoculum. Experimental flasks were incubated statically at 25 ± 1°C until the stationary growth phase. Growth was monitored by measuring the whole cell protein increase. Periodically, samples (0.5-ml) were taken from each flask, heated for 10 min with 0.05 ml of 1 N NaOH, and the protein content was then determined by the method of Bradford (1976) using human serum albumin as a standard.

Antimicrobial activity assay

Antimicrobial activity of the cell-free supernatants/extracts obtained after growth of the strain A-1 on medium with different carbon sources was evaluated by standard agar well diffusion assay (Bauer et al. 1966). Wells of 6 mm in diameter were punched in MPA plates seeded with test organisms. Three replicates were carried out against each of the test organisms. Overnight culture (50 µl) of the indicator cultures was added in the wells. Plates were then incubated overnight at 37°C for 24 h. At the end of the incubation time, the inhibitory activity was detected as a zone of clearing in the turbid agar around the wells containing antimicrobial activity (positive samples). The diameter of the clearing zones was measured for a semi quantitative determination of the concentration of the antimicrobial compound. The following test cultures were used: *B. subtilis* ATCC 6633, *Pseudomonas aeruginosa,*

Micrococcus sp., *Staphylococcus* sp., *Rhodotorula rubra* 6526 and *Xanthomonas oryzae*.

Extraction and characterization of the antimicrobial compounds

Lipid compounds were extracted twice from the cell-free supernatants of strain A-1 with chloroform/methanol (2:1 v/v). The pooled extracts were evaporated and the pellets were dissolved and used for thin-layer chromatography (TLC) analysis and antimicrobial activity tests. The isolated compounds were analyzed by TLC on silica gel 60 plates (G60, Merck, Germany) using chloroform–methanol–water (85:15:2 v/v/v) as a solvent system. Spots were revealed by spraying with specific reagents: orcinol for detection of sugar-containing compounds, and ninhydrin for detection of compounds containing free amino groups. Assay for sugar moiety was performed after hydrolysis of the glycolipid with 2 N H_2SO_4. The sugar component was identified by comparing the Rf-values with the standard sugars by TLC as described by Gesheva et al. (2010).

Results and discussion

Production of enzymes by Antarctic isolates

Antarctic isolates were tested for enzymatic production on solid media using specific substrates. The results revealed potential of the strains to produce a wide range of hydrolytic enzymes (Table 1). Thirty-five percents of the tested isolates showed proteolytic and amylolytic activity, and 60% of the isolates demonstrated lipolytic and urease activity. Most of isolates (about 80%) possessed phosphatase and cellulase activity, and only strain A-1 revealed RNase activity.

The ability of microorganisms to produce more than one type of enzymes allows them to effectively respond to the effect of various compounds entering the environment as a result of human activity. The enzymes produced by microbial cells play a key role in the degradation of a wide spectrum of organic polymers (Gianfreda and Rao 2004a, b; Mudryk and Skorczewski 2006). Extracellular enzymes initiate microbial remineralization of organic matter by hydrolyzing substrates to sizes sufficiently small to be transported across the cell membrane. Patterns of enzyme activities can be used to define the composition of organic matter sources in the ecosystems (Boschler and Cappenberg 1998).

Psychrophilic enzymes have a great exploitation potential (Zecchinon et al. 2000). It was reported that psychrophilic enzymes as proteases, amylases, and lipases participate in laundry detergents and bioremediation of waters following oil spills at cold temperatures (Rothschild and Manchinetti 2001).

Growth and enzyme production of strain A-1

Isolate marked as A-1 with a potential to synthesize all tested enzymes was chosen for further tests. The strain was cultivated in MSM with different carbon sources, and growth curves were prepared for each substrate (not shown). Values for growth (expressed as total protein content) at the stationary phase are presented in Fig. 1. As can be seen, the strain A-1 was able to use all tested substrates as carbon sources, and soy-bean flour and wheat bran were preferable substrates.

The carbon sources were examined for their effect on the production of hydrolytic enzymes by the strain A-1 (Table 2). Proteolytic and lipolytic activities were detected in all cell-free supernatants as they are not dependent on the used carbon source in the medium. It was found that only wheat bran and soy-bean flour were favourable substrates for amylase production; no RNase activity was detected in the cell-free supernatants from media with sunflower oil, waste frying oil, kerosene and phenanthrene each used as a sole carbon source.

Taxonomic determination of strain A-1

On MPA medium colonies of the isolate A-1 appeared pale yellow colour while on Küster agar they were sandy. The culture didn't form aerial mycelium and soluble pigment. Observations on cell morphology showed that the cells of strain A-1 are rods and cocci. The characteristics of the strain are summarized in Table 3. The isolate is Gram-positive, halophilic, grows aerobically and forms catalase, oxidase and urease. The strain A-1 possesses antibacterial and antifungal activity against Gram-positive bacteria and some yeasts. The culture didn't utilize arabinose, mannitol and inositol (Table 4). The morphology, biochemical, physiological features and whole-cell hydrolysate analysis have assumed that the strain A-1 belongs to the genus *Nocardioides*. In comparison with the described strains of this genus, the strain A-1 showed differences in utilization of carbon sources, enzyme and antibiotic formation (Prauser 1984, 1976; Collins et al. 1994; Yoon et al. 1997, 2004; Lawson et al. 2000; Hamamura and Arp 2000; Vasileva-Tonkova and Gesheva 2005).

Production of antimicrobial compounds by strain A-1

The cell-free supernatants obtained after growth of *Nocardioides* strain A-1 on different carbon sources were tested for antimicrobial activity towards some pathogenic

Table 1 Production of hydrolytic enzymes by Antarctic isolates tested on solid media with specific substrates

No	Isolates	Protease on skimmed milk	Proteaseon gelatin	RNAse	Amylase	Lipase	Phosphatase	Urease	Cellulase
A-1	Nocardioform	+ + +	+ + +	+	+	+	+	+ +	+
A-2	Coryneform	–	–	–	+ +	–	+	–	+ +
A-3	Coryneform	–	–	–	–	+ + +	+ +	+	+
A-4	Nonidentified	–	–	–	–	–	–	+	+
A-5	Coryneform	–	–	–	–	–	–	+	+
A-6	*Nocardia* sp.	–	–	–	–	–	–	+	+
A-6	Coryneform	+	–	–	–	–	+	–	–
A-8	Nocardioform	–	–	–	–	+	–	–	–
A-9	Coryneform	+ + +	+ + +	–	–	+ +	+	+	+
A-10	*Micromonospora* sp.	+ + +	+ +	–	+	–	+ +	–	+
A-11	Coryneform	+ +	+ +	–	–	+ +	+	+	–
A-12	Nonidentified	–	–	–	+ +	–	+	–	+
A-13	*Pantoea* sp.	+ + +	+ + +	–	–	+	+ +	–	+
A-14	Coryneform	+	+	–	+	+	+ +	+ +	+
A-15	Nocardioform	–	–	–	+ + +	+	+ +	+	+ +
A-16	Coryneform	–	–	–	–	+ +	+ +	+	+
A-17	Nocardioform	–	–	–	–	+ +	+ +	+	+

Symbols: –, negative result: no clear zone (or zone of growth); +, positive result: a clear zone (or zone of growth): +, zone of 1–2 mm; + + , zone of 3–5 mm; + + + , zone of 5 mm and more

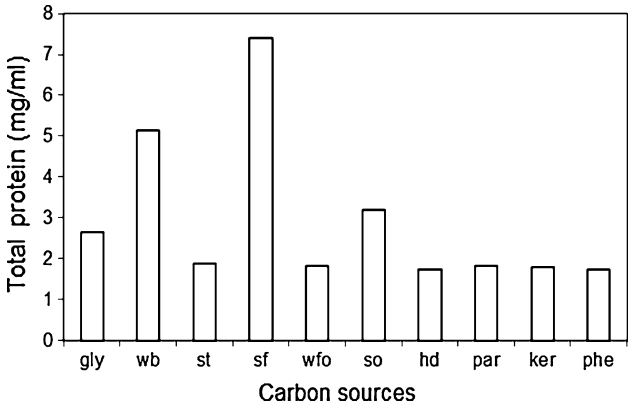

Fig. 1 Growth (expressed as total protein content) of *Nocardioides* sp. strain A-1 in mineral salts medium with different carbon sources: glycerol (gly), wheat bran (wb), starch (st), soy-bean flour (sf), waste frying oil (wfo), sunflower oil (so), hexadecane (hd), paraffin oil (par), kerosene oil (ker), phenanthrene (phe). Values are the mean of three separate experiments ± s.d. of within 5–10%

bacteria and yeasts. As can be seen in Table 5, almost all carbon sources favored production of antimicrobial compounds with activity against *B. subtilis* ATCC 6633, *Micrococcus* sp. and *X. oryzae*. The cell-free supernatants with glycerol, wheat bran and paraffin showed relatively high antimicrobial activity against *B. subtilis* ATCC 6633 with halo diameter in the range of 28–30 mm. Of importance is the presence of antimicrobial activity against plant pathogenic bacterium *X. oryzae* causing bacterial blight of rice- one of the most harmful diseases of rice. All tested carbon sources are favourable for production of compounds with antimicrobial activity against *X. oryzae* except sunflower oil, and starch and wheat bran were most suitable substrates giving inhibition halo diameter of 23 mm and 25 mm, respectively. The cell-free supernatants from media with glycerol, soy-bean flour and paraffin showed antimicrobial activity only against pathogenic *Staphylococcus* sp. No antimicrobial activity of all supernatants was detected against *Pseudomonas aeruginosa* and *Rhodotorula mucilaginosa* (known also as *R. rubra* producing enolase allergen).

It is known that the culturing process is important factor, because the antimicrobial substances may be produced mainly by the secondary metabolite pathway under the nutritionally limited conditions. The nutritional factors such as carbon and nitrogen source, ammonia, inorganic phosphate, and metal ions in the media may affect the production of secondary metabolites during cultivation of the microorganisms (Sanchez and Demain 2002; Gesheva et al. 2005).

Characterization of the antimicrobial compounds of strain A-1

The cell-free supernatants obtained after growth of *Nocardioides* sp. strain A-1 on glycerol, wheat bran, starch and paraffin that showed remarkable zones of inhibition

Table 2 Effect of different carbon sources on production of hydrolytic enzymes by strain A-1. Mean values from three determinations are given ± s.d

Carbon source	Halo (mm/diameter)					
	Protease		Protease	Amylase	Lipase	RNase
	Skimmed milk		Gelatin		Tween 80	
	48 h	72 h	72 h	48 h	72 h	48 h
Glycerol	25 ± 2	35 ± 1	25 ± 2	–	15 ± 2	16 ± 1
Wheat bran	23 ± 2	35 ± 2	26 ± 1	37 ± 1	20 ± 2	20 ± 1
Starch	24 ± 1	35 ± 1	30 ± 1	–	16 ± 2	13 ± 2
Soy-bean flour	25 ± 1	36 ± 1	26 ± 2	32 ± 1	22 ± 1	18 ± 3
Waste frying oil	24 ± 1	35 ± 1	30 ± 1	–	14 ± 2	–
Sunflower oil	18 ± 2	36 ± 1	30 ± 1	–	12 ± 3	–
Hexadecane	25 ± 2	35 ± 2	26 ± 2	–	15 ± 1	15 ± 1
Paraffin	23 ± 1	35 ± 2	28 ± 3	–	20 ± 2	16 ± 2
Kerosene	25 ± 1	35 ± 1	28 ± 1	–	15 ± 2	–
Phenanthrene	23 ± 1	34 ± 1	25 ± 2	–	19 ± 2	–

Table 3 Characteristics of *Nocardioides* sp. strain A-1

Properties	Strain A-1
Morphology	Rods, cocci
Colony colour	Sandy to pale yellow
Growth temperature range	10–40°C
Growth temperature optimum	20–25°C
NaCl tolerance	5%
Starch hydrolysis	–
Gelatinase protease	+
Lipase	+
Cellulase	+
Urease	+
Phosphatase	+
β-Lactamase	–
RNase	+
Oxidase	+
Catalase	+
Cell wall sugar	Galactose
Cell wall amino acid	LL-DAP
Antibiotic activity against:	
Bacillus subtilis ATCC 6633	+
Staphylococcus aureus 605P	+
Sarcina lutea	+
Escherihia coli	–
Saccharomyces cerevisiae	–
Candida tropicalis	+

Table 4 Utilization of carbon sources by *Nocardioides* sp. strain A-1

Source	Growth
Glucose	+
Fructose	+
Rhamnose	+
Arabinose	–
Xylose	+
Sucrose	+
Mannitol	–
Inositol	–
Glycerol	+
Starch	+
Sodium acetate	+

were selected for extraction of the antimicrobial compounds. The compounds in the organic extracts were developed by TLC and visualized with specific reagents.

Using of glycerol or starch in the fermentation medium favours formation of two blue spots on TLC sheets after orcinol/sulfuric acid staining with R_{f-1} 0.160 ± 0.014 and R_{f-2} 0.356 ± 0.005 indicating sugar-containing compounds, and one spot after staining with ninhydrin with R_f 0.328 ± 0.016 indicating compounds containing free amino groups. Organic extracts obtained after cultivation of the strain A-1 on wheat bran showed only one spot with R_f 0.104 ± 0.010 after orcinol/sulfuric acid staining. Application of paraffin as a carbon source revealed two spots: one spot with R_f 0.060 ± 0.010 after orcinol/sulfuric acid staining, and one spot with R_f 0.323 ± 0.004 after ninhydrin staining. Sugar presented in the glycolipids contained rhamnose which is confirmed by TLC of their acid hydrolysates. Therefore, the compounds antimicrobial produced by *Nocardioides* sp. strain A-1 are mainly glycolipids and/or lipopeptides depending on the used carbon source.

Glycolipids and lipoproteins are biosurfactants with increasing scientific, therapeutic and biotechnological interests (Kitamoto 2001; Herman and Maier 2002). Among the glycolipids, the best known are rhamnolipids,

Table 5 Antimicrobial activity (expressed as an inhibition halo, mm in diameter) of culture broths after growth of *Nocardioides* sp. strain A-1 in MSM supplemented with 2% of different carbon sources

Carbon source	Inhibition halo (mm in diameter)					
	Bacillus subtilis	*Xanthomonas oryzae*	*Staphylococcus* sp.	*Micrococcus* sp.	*Pseudomonas aeruginosa*	*Rhodotorula rubra*
Glycerol	30 ± 2	16 ± 1	18 ± 2	–	–	–
Wheat bran	30 ± 3	25 ± 3	–	18 ± 2	–	–
Starch	20 ± 1.5	23 ± 3	–	18 ± 1	–	–
Soy-bean flour	–	18 ± 2	12 ± 0.5	15 ± 2	–	–
Waste frying oil	18 ± 2	15 ± 1	–	20 ± 2	–	–
Sunflower oil	20 ± 1	–	–	18 ± 2	–	–
Hexadecane	–	16 ± 1	–	14 ± 1	–	–
Paraffin oil	28 ± 3	18 ± 2	10 ± 1	16 ± 1	–	–
Kerosene oil	–	15 ± 1	–	–	–	–
Phenanthrene	20 ± 1	16 ± 1	–	11 ± 1	–	–

Values are the average of three replications ± s.d

trehalosolipids and sophorolipids. They include rhamnolipids produced predominantly by *Pseudomonas* strains (Abdel-Mawgoud et al. 2010), trehalosolipids from *Rhodococcus* spp. (Franzetti et al. 2010) and sophorolipids from *Candida* spp. (Shah and Badia 2007). Rhamnolipids have been shown to display antibacterial and antifungal activities (Arutchevi and Doble 2010; Vatsa et al. 2010; Sarin et al. 2011). Lipopeptides are the most frequent antibiotic compounds produced by *Bacillus* spp. exhibiting a wide antimicrobial spectrum and exceptional surfactant activities. Cyclic lipopeptide surfactin is a very powerful surfactant commonly used as antibiotic. It is one of the 24 types of antibiotics produced by *B. subtilis* strains (Ahimou et al. 2000). Other lipopeptide-type surfactins are produced by *Bacillus polyfermenticus* (Kim et al. 2009) and *Bacillus amyloliquefaciens* (Vitullo et al. 2011). Iturins from *B. subtilis* and lichenysins from *Bacillus licheniformis* are also reported that are similar in structural and physicochemical properties to the surfactin (Ahimou et al. 2000; Yakimov et al. 1995).

The actinomycetes are rare in Antarctic soils (Greenfield 1981; Broady et al. 1987; Vasileva-Tonkova and Gesheva 2004, 2005, 2007; Gesheva 2005, 2009a, 2010; Gesheva et al. 2010). Data about Antarctic microorganisms producing antimicrobial compounds are scant (Siebert et al. 1996; Giudice et al. 2007; Gesheva 2009a, b, 2010). The group of nocardioform actinomycetes is characterized by a wide degradative potential that allows them to utilize a wide range of compounds including persistent ones or to transform them into metabolites of practical interest (Vasileva-Tonkova and Gesheva 2004, 2005; Solyanikova et al. 2008). This feature makes the representatives of this group very promising for application in various fields of industry. Isolation and study of active strains with broad substrate specificity is of the first priority for investigators. The results obtained showed that the halophilic Antarctic *Nocardioides* sp. strain A-1 present a great potential for biotechnological, biopharmaceutical and biocontrol applications as a source of industrially important enzymes and antimicrobial/antifungal compounds. Antibiotic compounds produced by *Nocardioides* sp. strain A-1 exhibited antibacterial activities towards Gram-positive and Gram-negative bacteria, especially *X. oryzae* causing bacterial blight, major disease in rice producing countries where high-yielding rice cultivars are often highly susceptible to it. It is a vascular disease resulting in tannish-grey to white lesions along the leaf. In infested fields, bacterial blight can cause yield losses up to 50%. When it infects at the seedling stage, it causes a syndrome known as kresek, which lead to nearly complete crop loss. Because of that the finding of the compounds produced by *Nocardioides* sp. strain A-1 with antibiotic properties against *X. oryzae* opens a perspective for future application in the agriculture for plant protection.

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