SUPPLEMENTARY MATERIAL

A new chitinase-producer strain *Streptomyces glauciniger* WICC-A03: isolation and identification as a biocontrol agent for plants phytopathogenic fungi

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Abstract

The objective of this study discusses the isolation and identification of a new *Streptomyces* highly active chitinase producer. Fifteen strains were isolated from Malaysian soil samples. The isolate WICC-A03 was found to be the most active chitinase producer. Its antifungal activity was evaluated against many phytopathogens. The identification of WICC-A03 using phenotypic and genotypic methods strongly indicated that strain WICC-A03 belonged to the genus *Streptomyces* and showed similarity (91%) with *Streptomyces glauciniger*. Thus, it was given the suggested name *S. glauciniger* WICC-A03 with accession number: JX139754. WICC-A03 produces extracellular chitinase in a medium containing 1.5% colloidal chitin in submerged culture on 144 h. The produced enzyme was partially characterised and its molecular weight of 50 kDa was obtained by using SDS-PAGE. The present study indicates that WICC-A03 is a potential chitinase producer for biocontrol of plant pathogens. Further experiments are being carried out to optimize medium composition and cultivation conditions under lab and bioreactor scale.

Keywords: Isolation; Antimicrobial activity; Phenotypic and genotypic identification; Chitinase; *Streptomyces glauciniger* WICC-A03; Biocontrol.

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3 Experimental

3.1. Organism and cultural conditions

For enrichment of chitinase-producing microorganisms, a mineral medium containing colloidal chitin as the sole carbon and energy source was used and incubated at 28 °C. The temperature was kept constant throughout the incubation period. The sample that was used for this experiment was obtained from Malaysian soil samples where 5 grams of soil were added into 50 ml distilled water. After the sample was incubated for 1 h, it was spread onto a chitinase detection medium that contained the following (g L^{-1}): 1 colloidal chitin, 0.7 KH_2PO_4 , 0.3 K_2HPO_4 , 4 NaCl, and 0.5 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ that was solidified with 2% agar. Clear halos were observed around the colonies after incubation for 7 d. Colonies showing zones of clearance against the creamy background were regarded as chitinase-producing and restreaked until pure cultures were obtained.

Among the seven colonies, a colony that showed predominant chitinase activity was selected and grown in a chitinase production medium that was made up of (g L^{-1}): 15 colloidal chitin; yeast extract 0.5; KH_2PO_4 , 1.36; $(\text{NH}_4)_2\text{SO}_4$, 1; and $\text{MgSO}_4 \cdot 6\text{H}_2\text{O}$, 0.3 at 28°C for 144h. The culture was harvested, filtered and centrifuged. The supernatant was used for further chitinase purification (Kyoung-Ja et al., 2003).

3.2. Phenotypic identification method

The cultural, morphological, physiological and biochemical characteristics of WICC-A03 strain were assessed following the guidelines adopted by the *International Streptomyces Project* (ISP) (Shriling & Gottlieb, 1966). Carbohydrate utilization was determined based on its growth on the ISP-9 medium supplemented with 1% carbon source at 28°C. The diaminopimelic acid (LL-DAP) isomers in the cell wall were analyzed as described by Lechevalier & Lechevalier (1970). The media composition and the cultivation conditions were implemented as described by Shriling & Gottlieb (1966).

3.3. Genotypic identification method

3.3.1. *Streptomyces* genomic DNA isolation, PCR amplification and sequencing

The total genomic DNA preparation was conducted using the method of Lee et al. (2003). The amplifications were performed in a GeneAMP PCR System 9700 (PE Applied Biosystems, PerkinElmer, Ohio, USA). The universal primers used were StrepR (5'-ACGTGTGCAGCCCAAGACA-3', forward) and StrepF (5'-ACG TGT GCA GCC CAA GACA-3', reverse) using the Biolegio BV software (Biolegio, Nijmegen, the Netherlands) (Rintala et al., 2001). The PCR reaction mixture conditions were as described in Awad et al. (2009). The PCR reaction mixture conditions (25µL) contained PCR beads (Amersham Bioscience Europe GmbH, Freiburg, Germany), 0.5µL from StrepR and StrepF respectively, 2 µL of template genomic DNA, and deionized water filled up until the final 25µL.

Amplification was performed with an initial denaturation step of three min at 94 °C and then 35 cycles (60 sec at 94 °C and 30 sec at 59 °C for primer annealing; and 60 sec at 72 °C for primer extension). This was kept at 72 °C for 7 min to complete the extension. The PCR products were done by Agarose gel electrophoresis and photographed using a gel documentation system (Alpha-Imager 2200, CA, USA) according to Awad et al. (2009) and were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

The sequencing of the purified PCR products was conducted using an ABI Prism 377 DNA Sequencer (PerkinElmer, Ohio, USA) with the Big Dye® Terminator v3.1, the Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, USA) and the universal primers listed above in the Gene Analysis Unit (Cornell University, NY, USA, Sequencing Facility Center).

3.3.2. Phylogenetic analysis and tree construction

The phylogenetic data were obtained by aligning the nucleotides of various 16S rRNA retrieved by the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) using the CLUSTAL W programme with standard parameters. The classifier was trained on the new and phylogenetically consistent higher-order bacterial taxonomy (Ribosomal Database Project, RDP Classifier) that was proposed by Wang et al. (2007) (<http://rdp.cme.msu.edu/classifier/classifier.jsp>). The phylogenetic and molecular evolutionary analyses were conducted using BioEdit.

3.3.3. Nucleotide sequence ID

The nucleotide sequence of the 16S rRNA gene of strain WICC-A03 has been deposited in GenBank under the accession number: JX139754.

3.4. Secondary structure prediction and restriction site analysis

The RNA secondary structure of the isolate WICC-A03 was predicted as described by Brodsky et al. (1995) using the GeneBee online software (http://www.genebee.msu.su/services/rna2_reduced.html) with the greedy method. The restriction sites of the WICC-A03 DNA were analyzed by NEBcutter V2.0 (<http://tools.neb.com/NEBcutter2/>), as described by Vincze et al. (2003).

3.5. Isolation of the fungal pathogens and antifungal activity assay

To obtain pure cultures of fungi species, the single-spore technique was used (Nelson et al., 1983). The fungal pathogens used in the study were *Fusarium oxysporium*, *Fusarium solani* and *Cladosporium inacrocanpum*. These were isolated from dragon fruit panicles in an infected field in Johor Bahru, Malaysia. The culture and microscopic features of these fungi were analyzed and identification was based on current universal keys for fungi (Samson et al., 2000). Each fungus was maintained on Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) to culture the fungi at 25°C for 48 h. The bioassay method is in accordance to the agar-well diffusion technique (Cooper, 1972). Each treatment was replicated three times and the results were expressed as the mean of these three replicates.

3.6. Antimicrobial susceptibility

Sensitivity to the antibiotics was tested in the Muller Hinton medium (Difco, NJ, USA) using the disc diffusion method. A sterilized filter paper disc impregnated with an antibiotic was placed on the spread cells and incubated overnight. Ampicillin, chloramphenicol, tetracycline hydrochloride and streptomycin sulphate (Oxoid Limited, Hampshire, UK) were compared. All antibiotics were tested at concentrations of 25, 50 and 100 µg mL⁻¹.

3.7. Light microscopic examination for antifungal activity

For understanding the antifungal mechanism, *Fusarium oxysporium* was treated with sterile water and purified enzyme for 48 hours separately, and then the mycelium was observed under light microscopic with magnification power of 400 times (Gao et al., 2008).

3.8. Chitinase enzyme assay

Chitinase activity was assayed with colloidal chitin as the substrate. Enzyme solution (0.5 mL) was added to 1 mL substrate solution, which contained 0.3 % colloidal chitin in a potassium phosphate buffer (20 mM, pH 6.0). The mixture was incubated at 45 °C for 1 h. After centrifugation, the amount of reducing sugar produced was measured by the method of Imoto & Yagashita (1971). The reducing sugar levels were determined relative to N-acetyl-β-D-glucosamine standards of 10-150 µg mL⁻¹. One unit of chitinase activity is defined as the amount of enzyme producing 1 µmol reducing sugar per minute at 45 °C. Substrate and enzyme blanks were also prepared in which enzyme or substrate was replaced with buffer. All measured values of absorbency were corrected for the appropriate blank absorbencies (Kyoung-Ja et al., 2003).

3.9. Enzyme characterisation and determination of molecular weight.

A 500 µl supernatant of culture broth were precipitated with TCA. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described in Laemmli (1970) and Wang et al. (2001) using 5% stacking and 15% resolving gels. A MW-Polypeptide SDS-PAGE Molecular Weight Standard Catalog Number 161-0326 (Biorad Hercules, CA, USA) and BSA (66 kDa standard) were used to provide molecular weight standards. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250.

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Table S1. Antimicrobial activity of isolate no.WICC-A03 crude extract showing the inhibition zone (I.Z) diameter by mm using agar well diffusion method

Bacteria						Fungi			
G+ve			G-ve			Unicellular	Filaments fungi		
<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Enterobacter aerogenes</i>	<i>Salmonella enterica</i>	<i>Pseudomonas aerogenos</i>	<i>Escherichia coli</i>	<i>Candida albicans</i>	<i>Fusarium oxysporium</i>	<i>Cladosporium macrocarpum</i>	<i>Fusarium solani</i>
14.5	12.5	15.0	-	15.0	-	15.0	20.0	15.0	15.0

I.Z= inhibition zone diameter by mm, G^{+ve} = Gram positive, G^{-ve} = Gram negative

Table S2. Antimicrobial susceptibility of strain WICC-A03 using the agar disc diffusion method.

Antibiotic	Code & Conc.	Inhibition Zone (mm)
Gentamycin	CN (10 µg)	12
Tetracycline	TE (30 µg)	15
Chlromphenicol	C (30 µg)	00
Streptomycin	S (10 µg)	24
Vancomycin	VA (30 µg)	19
Blank Disc	-	00

1 Table S3. Physiological, morphological and biochemical properties of WICC-A03 strain.

Characteristics	Results
Spore chains	Straight
Spore surface	Smooth
Color of aerial mycelium	White
Melanin production	None
Action of milk	Positive
Nitrate reduction	Negative
Gelatin liquefaction	Positive
Starch Hydrolysis	Positive
Cell wall	LL-Diaminopimelic acid
D-glucose	+
D-xylose	++
L-arabinose	+
L-rhamnose	+
D-fructose	+
D-galactose	+
Raffinose	++
D-mainitol	-
Meso-inositol	-
Salicin	-
Sucrose	++
Maltose	+
Lactose	+

2 +=Modrate growth, ++= Good growth, -= no growth

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1 Table S4. Cultural characteristics of strain WICC A03 at 7-14 days

Medium No.	Growth	Color of		Soluble Pigment
		AM	SM	
ISP2	Moderate	White gray	Creamy	-
ISP3	Moderate	White	Creamy	-
ISP4	Abundant	White gray	Yellowish	-
ISP5	Abundant	White	Creamy	-
Bennett's	Abundant	White	Creamy	-
Czapek's	Abundant	White gray	Yellowish	-
Glucose asparagine	Moderate	White	Velvety	-
ISP7	fair	White	velvety	-
Nutrient agar	Fair	White	Creamy	-

2 AM = aerial mycelium, SM = Substrate mycelium

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6 (A)

7 (B)

8 Figure S1. *Fusarium oxysporium* treated with sterile distilled water (A) and the same strain was treated with crude chitinase for 48h (B).

(A)

(B)

Figure S2. The morphology of WICC-A03. Spore chains of the Rectiflexibles type are shown at 2,000X (A) and the spore surface is shown as smooth at 25,000 X (B) using Bennett's agar medium at 28 °C.

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Figure S6. Restriction sites of the strain WICC-A03 were predicted using NEBcutter.

Figure S7. SDS-PAGE of crude chitinase from the culture broth of strain WICC-A03