

## A STUDY ON THE BACTERIAL STRAIN BRI 1 AS A CHITINOLYTIC MICROORGANISM ISOLATED FROM MANGROVE SOIL

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**ABSTRACT.** Sabah with its mega-biodiversity is believed to harbour various chitinolytic microorganisms which exhibit optimal chitinase activities at the local ambience. Bacterial strain BRI 1 was recovered from mangrove soil in Kota Belud, Sabah using Chitinase Detection Agar, pH6.5. The strain BRI 1 was placed under the genus *Streptomyces* as its physical morphology showed typical streptomycete appearance on solid medium. This was further supported with the analysis of its amplified ~1.5kb 16S rDNA fragment in which it showed close relation to *Streptomyces* sp. Amplification of family 18 chitinase gene generated an amplicon of 397 bp. Similarly, amplification of family 19 chitinase gene resulted in 342 bp amplicon. Chitinase identity of both amplicons were confirmed in which they showed similarity to chitinase genes from *Streptomyces* sp.. Crude chitinase activity of BRI 1 showed 8.61 Unit, a three-fold higher than the activity exhibited by *Streptomyces griseus* which only showed 2.54 U in a triplicate assay using chitin azure as enzymatic substrate. The data resulted in this report serve as a platform for further investigations involving characterization of the chitinases and manipulation of the chitinase genes.

**KEYWORDS.** *Streptomyces*, chitinase gene, chitin azure.

### INTRODUCTION

Chitin is the second most abundant biopolymer on earth after cellulose. It is degraded and recycled in the biosphere by chitinases (EC 3.2.1.14). Chitinases belong to either family 18 or family 19 in the superfamily of glycosyl hydrolase (Henrissat, 1991; Henrissat & Bairoch, 1993). Chitinases are produced by various organisms that include the eukaryotes, prokaryotes and viruses. Chitinases secreted by bacteria are mostly from the family 18 (Jollès & Muzzarelli, 1999). However, there are increasing findings reporting the detection of both families of chitinase genes in bacteria especially from the actinomycetes (Watanabe *et al*, 1999). Chitinases and chitinase genes have been isolated and characterized from various organisms. Transgenic plants harbouring foreign chitinase genes have been developed. The transgenic plants exhibit enhanced resistance towards fungal and insect attacks (Kishimoto *et al*, 2002). In addition, attempts to develop potent biopesticides co-expressing chitinase and toxin are in progress. Engineered toxin and chitinase producing microorganisms may increase lethality towards insects as the chitinase perforates the mid-gut lining of the insects allowing efficient contact of toxin with target cells (Sirichotpakorn *et al*, 2001). In this report, a bacterial strain, BRI 1 isolated from mangrove soil sampled at Kota Belud was explored in terms of its physical morphology, chitinase genes and chitinase activity.

## MATERIALS AND METHODS

### Bacterial strain and media

The bacterial strain BRI 1 was recovered from mangrove soil sampled from Sungai Merajah at the district of Kota Belud, Sabah, Malaysia. The strain was recovered using Chitinase Detection Agar (CHDA) (Chih *et al.*, 2002) containing 20g/L agar, 30g/L colloidal chitin, 0.65g/L Na<sub>2</sub>HPO<sub>4</sub>, 1.5g/L KH<sub>2</sub>PO<sub>4</sub>, 0.25g/L NaCl, 0.5g/L NH<sub>4</sub>Cl, 0.12g/L MgSO<sub>4</sub>, 0.005g/L CaCl<sub>2</sub>. Cycloheximide at the final concentration of 50µg/mL was added to the media. The pH of the medium was adjusted to pH6.5, an average pH of the sampled soil. Working stock of BRI 1 was maintained at 28°C.

### Microbial characterization

BRI 1 was cultured on a thin layer of CHDA and its morphologies at the macroscopic and microscopic level were observed. Gram staining was performed to determine its Gram type. The identity of its 16S rDNA was determined in which PCR amplification of the 16S rDNA sequence was carried out using PCR primer pair, p27f: 5' AGAGTTTGATCMTGGCTCAG 3' and p1525r: 5' AAGGAGGTGWTCCARCC 3' (Chun & Goodfellow, 1995) with a cycling program of 1 cycle at 98°C for 1 minute, 25 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes. A final extension was performed with 1 cycle of 72°C for 10 minutes.

### Enzyme and protein assay

A filtered cultured broth of chitinase detection medium was used for chitinase assay. A volume of 0.5mL of chitinase is incubated with 0.3% (w/v) chitin azure (Sigma C3020) in a total enzymatic reaction volume of 2.5mL. The reaction was buffered with 50mM sodium phosphate buffer to achieve pH6.5. The assay was performed at 28°C for 48 hours before 2mL of 2.4M potassium phosphate buffer (pH9.0) was added to stop the enzymatic reaction. Protein quantification was carried out using PRO-MEASURE solution according to the manufacturer's instruction. One unit of chitinase activity is defined as a change in the optical density value at 575nm of 1.0 by one milligram of chitinase per 24 hours of enzymatic assay at 28°C, 50mM sodium phosphate buffer, pH6.5.

### Isolation of chitinase genes

Family 18 group A chitinase gene was amplified using PCR primer pair, GA1F: 5' CGTCGACATCGACTGGGARTDBCC 3' and GA1R: 5' ACGCCGGTCCAGCCNCKNCCRTA 3' (Williamson *et al.*, 2000) with PCR cycling program of 1 cycle of 94°C for 5 minutes, 30 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute. A final extension was performed with 1 cycle of 72°C for 10 minutes. Family 19 chitinase gene was amplified using PCR primer pair, F19actF: 5' AAGCTCGCSGCTTCCTSGC 3' and F19actR: 5' GCACTCGAGSGCGCCGTTGAT 3' (Kawase *et al.*, 2004) with PCR cycling program of 1 cycle of 98°C for 2 minutes, 30 cycles of 98°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. A final extension was performed with 1 cycle of 72°C for 10 minutes. PCR products were purified using Qiagen Gel Extraction Kit according to the manufacturer's instruction. The nucleotide sequences of the purified PCR products were determined with ABI PRISM @377 DNA sequencer using BigDye@Terminator v3.1 cycle sequencing kit.

## RESULTS AND DISCUSSION

### Morphological characteristics of the bacterial strain BRI 1

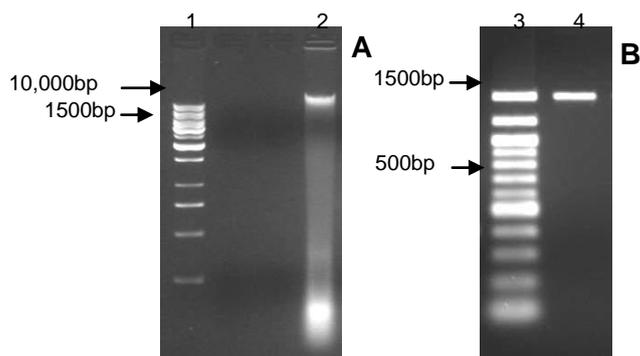
The bacterial strain BRI 1 showed actinomycetic characteristics (Fig. 1). It sporulates when grown on CHDA where it produces creamy white spores and halo around its colonies. Observation at the microscopic level revealed that both aerial and substrate mycelia present. The aerial mycelia consist of sporophores in the *rectiflexibile* organization. Gram staining showed the retaining of purple stain of crystal violet indicating that the strain is a Gram-positive bacterium.



**Figure 1. Morphology of the bacterial strain BRI 1. (A) The strain sporulating on CHDA, (B) substrate mycelia, (C) Sporophore with *rectiflexibile* organization.**

### Analysis of the 16S rDNA

PCR amplification of the 16S rDNA generated an amplified PCR product of around 1.5kb (Fig. 2). The 5' terminal sequence of 632bp was compared to other nucleotide sequences in the nucleotide sequence databases. Similarity to other 16S rDNA sequences was performed using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST/>). BLASTn searches of the partial 16S rDNA fragment of the strain resulted in sequence similarity to 16S rDNA fragments from *Streptomyces* sp. (Fig. 2 & Table 1). This further confirmed that BRI 1 is an actinomycete.



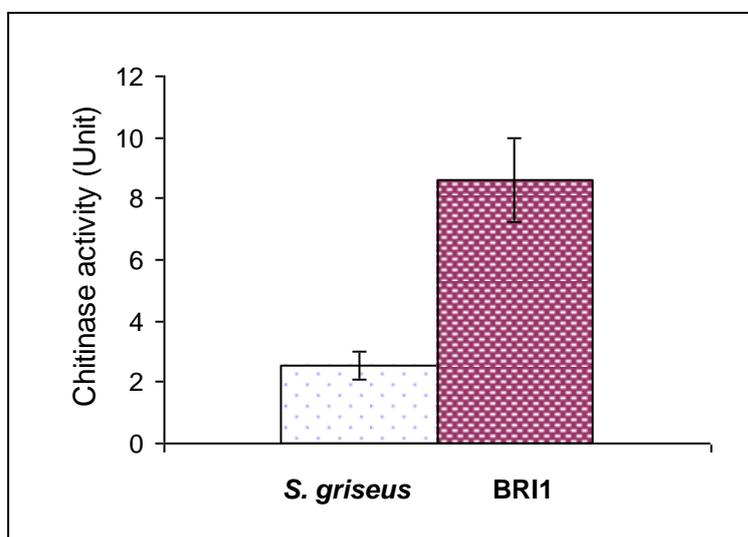
**Figure 2. Gel picture A: genomic DNA of BRI 1 was electrophoresed on 1% agarose using 1x TBE buffer at 85V for 45 minutes. Lane 1= 1kb DNA ladder (Fermentas), lane 2= genomic DNA of BRI 1. Gel picture B: The 16S rDNA amplicon of BRI 1 was electrophoresed on 1% agarose gel, using 1x TBE at 80V for 60 minutes. Lane 3= 100bp DNA marker (NEB), lane 4= 16S rDNA fragment of BRI 1.**

**Table 1. Some 16S rDNA sequences that share high similarities with BRI 1 16S.**

Sequences producing significant alignments	Score (Bits)	E-value	Identities
<i>Streptomyces alboniger</i> 16S ribosomal RNA gene	1114	0.0	597/606 (98%)
<i>Streptomyces alboniger</i> strain JCM 4309 16S ribosomal RNA gene	1114	0.0	597/606 (98%)
<i>Streptomyces tauricus</i> gene for 16S rRNA	1074	0.0	592/606 (97%)
<i>Streptomyces hygroscopicus</i> subsp. <i>hygroscopicus</i> gene for 16S rRNA	1074	0.0	592/606 (97%)
<i>Streptomyces peruviansis</i> partial 16S rRNA gene, type strain DSM 40592	1072	0.0	583/596 (97%)

### Analysis of chitinase assay

The crude chitinase activity of the bacterial strain BRI 1 was compared with purified enzymes from *Streptomyces griseus* (Sigma C6137) based on the assay recommended by Sigma using Chitin Azure (C320) as the enzymatic substrate. The crude chitinase activity of the bacterial strain BRI 1 showed 8.61 Unit (U), an activity of three folds higher compared to that of *S. griseus* which only showed 2.54 U in the triplicate assay (Fig. 3). This suggests that the purified chitinase activity of strain BRI 1 might be more than three folds to those of the *S. griseus*.

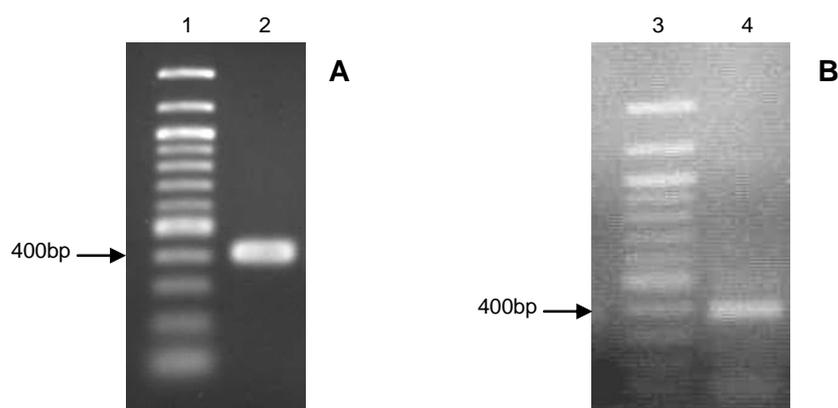


**Figure 3. A three-fold higher chitinase activity was detected when a comparison was made between crude chitinase preparation from BRI 1 and chitinase from *S. griseus*. Error bar represents standard deviation.**

### Analysis of chitinase genes

Both families of chitinase genes were detected in the genome via PCR. A fragment of 397bp of the family 18 chitinase gene was generated, sequenced and subjected to BLASTn analysis (Fig. 4). The nucleotide-nucleotide sequence analysis revealed that the chitinase gene

fragment showed similarities to many family 18 chitinase genes fragments from *Streptomyces* sp. (Table 2). On the other hand, a fragment of 342 bp of family 19 chitinase gene was generated, sequenced and subjected to BLASTn analysis (Fig. 4). Similarly, the gene fragment showed similarities to many family 19 chitinase genes from *Streptomyces* sp. (Table 3). A deduced amino acid sequence of the fragment revealed the presence of VAWKTGLWYWN that correspond to the chitinase motif [LIVM]-[GSA]-F-x-[STAG](2)-[LIVMFY]-W-[FY]-W-[LIVM]. Based on the BLASTn result, none of the sequences in the nucleotide database are identical to the sequences of the amplicons generated in this study. This suggest that BRI 1 harbours novel family 18 and family 19 chitinase genes.



**Figure 4.** Gel picture A: The amplicon of family 18 chitinase gene generated from BRI 1 was electrophoresed with 1.3% agarose gel using 1x TBE buffer at 80V for 60 minutes. Lane 1= 100bp DNA marker (NEB) and lane 2= amplicon of family 18 chitinase gene from BRI 1. Gel picture B: The amplicon of family 19 chitinase gene generated from BRI 1 was electrophoresed with 1.2% agarose gel using 1x TBE buffer at 85V for 45 minutes. Lane 3= 100bp DNA ladder (NEB) and lane 4= the amplicon of family 19 chitinase gene from BRI 1.

**Table 2.** Some chitinase sequences sharing high similarity with family 18 chitinase gene fragment of BRI 1.

Sequences producing significant alignments	Score (Bits)	E-value	Identities
<i>Streptomyces roseoflavus</i> chitinase C (chiC) gene	234	1e-59	166/182 (91%)
<i>Streptomyces avermitilis</i> putative chitinase C precursor	232	6e-59	228/265 (86%)
<i>Streptomyces coelicolor</i> A3(2) chitinase (putative secreted protein)	212	5e-53	229/267 (85%)
<i>Streptomyces coelicolor</i> gene for ChiD	212	5e-53	229/267 (85%)
<i>Streptomyces thermoviolaceus</i> chi40 gene for chitinase	188	8e-46	125/135 (92%)

**Table 3. Some nucleotide sequences that produced significant alignments with family 19 chitinase gene fragment of BRI 1.**

Sequences producing significant alignments	Score (Bits)	E-value	Identities
<i>Streptomyces coelicolor</i> A3(2) complete genome; chitinase	333	2e-89	284/320 (88%)
<i>Streptomyces coelicolor</i> gene for ChiF, partial cds	333	2e-89	284/320 (88%)
<i>Streptomyces coelestis</i> gene for chitinase 19-2, partial cds, strain:ISP5421	329	3e-88	276/310 (89%)
<i>Streptomyces coelicolor</i> gene for chitinase 19-1, partial cds, strain:A3-2	315	4e-84	275/311 (88%)
<i>Streptomyces lividans</i> gene for chitinase 19-1, partial cds, strain:66	313	2e-83	274/310 (88%)

## CONCLUSION

The bacterial strain BRI 1 is concluded to belong to the genus *Streptomyces* based on morphological observation and analysis of its 16S rDNA fragment. BRI 1 harbour both family 18 and family 19 chitinase genes. Local alignment of the fragments with other nucleotide sequences in the nucleotide sequence databases revealed that their chitinase identity showed close relation to chitinase genes from *Streptomyces* sp.. Crude chitinase activity of BRI 1 was determined to be three-fold higher than that from *Streptomyces griseus* (Sigma C6137). Further investigations are open for the characterization of the chitinases and manipulation of its chitinase genes.

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