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Identification And Characterisation of Chitinolytic Strains of *Stenotrophomonas Maltophilia*, Isolated From Mentha and Ocimum Plants of Saudi Arabia

Biological Science

KEYWORDS : Chitinase, anti-fungal activity, 16S rDNA sequencing and Stenotrophomonas maltophilia.

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ABSTRACT

The research screened chitinolytic rhizobacteria isolated from Mentha and Ocimum plants from Jeddah, Mecca and Al-Madinah Al-Munawaraha of Saudi Arabia to determine their chitinase activity in degrading chitin on colloidal chitin media. Bacterial isolates evaluated for chitin degradation on chitinase detection agar after 7,10 and 15 days at 30°C. Out of 100 colonies nine isolates showed halo zone around their colonies. Isolates identified as Stenotrophomonas maltophilia by 16S rRNA gene sequencing. The research aim is to screen chitinolytic rhizobacteria isolated from different plants at some localities of Kingdom Saudi Arabia to determine their chitinase activity in degrading chitin of shrimp. In addition, the highest chitinase production isolates will detect by PCR technique and identified morphology and 16s rDNA sequences. This study summarizes the advances of using chitinase production from chitinolytic rhizobacteria with special advantage to be biological fungicide and insecticide to control pathogenic fungi and insect pests.

Introduction

The application of pesticides must provide significant level of pest control and must be also economical. There are adverse consequences of using pesticides to the environment and public health (Hall, 1991).

Control of plant pathogen is one of the main goals of using chitinases as biocontrol agents. Cultures of chitinolytic rhizobacteria, synthesized by chitinases and their chitinase genes all proved to be efficient biological agents (Brurberg *et al.,* 2000).

Chitin is a linear β -(1,4)-linked N -acetylglucosamine (GlcNAc) polysaccharide. It is found in structural components of fungi and insects. Chitin can be enzymatically degraded by chitinase. This enzyme has been successfully used as a biological fungicide and insecticide to control phytopathogenic fungi and insect pests. This makes chitinases inhibitors attractive targets for possible antifungal and insecticidal agents.

Stenotrophomonas maltophilia strains have been used for biological control of plant diseases caused by fungus due to its ability to synthesize chitinase as well as a number of several other metabolites (Minkwitz and Berg, 2001). It has been recognised that this bacteria has the ability to excrete chitinase. This is responsible for controlling pathogenic fungi. The activity of chitinolytic enzymes makes them a potent anti chitin-containing pathogens biological control agents. For this reason, multiple studies focused on the mechanism of action of chitinolytic enzymes (Shaikh and Deshpande, 1973; Carr and Klessig, 1989; Linthorst, 1991).

MATERIAL AND METHODS

Screening of chitinolytic bacteria

Rhizospheric region micro flora of "Mentha" and "Ocimum" plants of soil samples isolated from Jeddah, Mecca and Al-Madinah Al-Munawaraha of Saudi Arabia.

Preparation of Colloidal Chitin

Prepared as described by (Wen *et al.* 2002).

Preparation of Chitin Plates

As described by (Jholapara *et al.* 2006) Colloidal chitin agar medium (CCA) was containing 1.25% Colloidal chitin in Minimal medium M9. M9 plates were prepared by mixing equal volume of 2x M9 with colloidal chitin. The plates were incubated at 30°C for 7,10 and 15 days.

PCR amplification of 16S rRNA gene

Genomic DNA isolated following GeneJET Genomic DNA Purification Kit. The universal primers were by (Stravato and Cappelli, 2001), (Forward Primer) 5′-AGAGTTTGATCCTGGCTCAG -3′ and (Reverse primer) 5′- AAGGAGGTGATCCAGCCGCA -3′. The reaction conditions follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min; followed by a final extension at 72°C for 10 min.

RESULTS AND DISCUSSION

Screening of chitinolytic bacteria

Rhizospheric region micro flora of "Mentha" and "Ocimum" plants used to isolated chitinolytic bacteria. The soil samples were isolated from three different locations of Saudi Arabia, Jeddah, Mecca and Al-Madinah Al-Munawaraha. The samples were serially diluted with physiological saline (NaCl 8.5 g/L) until the dilutions of 10^7. Dilutions were inoculated on colloidal chitin agar medium (CCA) containing 1.25% colloidal chitin. The bacteria isolate exhibiting a clear zone (as a sign for chitin degradation). Bacteria picked up and applied for purification. **Figure 1** and **Table 1** indicated that out of 100 bacterial isolates, only nine isolates have been selected depending on the highest producing of halo zones. The qualitative analysis of chitinolytic activity of the selected bacterial isolates was conducted by plate screening method on colloidal chitin agar medium plates (Wen *et al.* 2002). All the plates were incubated at 30°C for 7, 10 and 15 days. A clear zone around a growing colony indicated chitin degradation and was measured using chitin degradation index equation (DI) as following:

DI = Colony diameter + Halo zone diameter/ Colony diameter

All the observations were recorded. Strains developing clear zone around their colonies could easily be identified as chitin degradations.

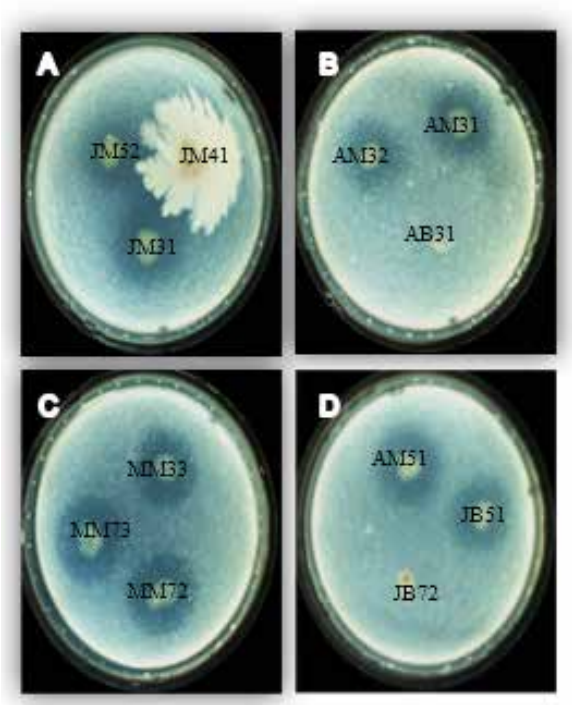

Figure 1: Clearing zone of chitinase activity observed after 7 days of inoculation in chitinase detection medium supplemented with shrimp colloidal chitin (1.25%). A represents JM52, JM31 isolates showed clear zone of chitin degradation, while JM41 isolate was unable to degrade chitin on colloidal chitin agar medium. B represents AM31, AM32 isolates showed clear zone of chitin degradation while AB31 isolate was unable to degrade chitin on colloidal chitin agar medium. C represents MM33, MM73 and MM71 isolates showed clear zone of chitin degradation on colloidal chitin agar medium. D represents AM51, AB51 isolates showed clear zone of chitin degradation while JB72 isolate was unable to degrade chitin on colloidal chitin agar medium.

Isolates	Colony diameter (cm)	Zone diameter (cm)	(DI)	Colony diameter (cm)	Zone diameter (cm)	(DI)	Colony diameter (cm)	Zone diameter (cm)	(DI)
	After 7 days			After 10 days			After 15 days		
JM31	0.05	1.3	3.6	0.06	1.7	3.8	0.08	0.02	3.5
JM52	0.04	1.2	4.0	0.05	1.6	4.2	0.06	1.8	4.0
JB51	0.05	1.2	2.9	0.06	1.7	3.8	0.07	1.9	3.71
AM31	0.05	1.2	2.9	0.08	1.4	2.75	1	1.7	2.7
AM32	0.04	1.3	4.25	0.05	1.7	4.4	0.06	2.0	4.3
AM51	0.03	1.1	4.6	0.05	1.6	4.2	0.08	1.8	3.25
MM33	0.04	1.5	4.75	0.06	1.7	3.8	0.08	2.0	3.5
MM72	0.03	1.7	6.6	0.06	1.9	4.1	0.09	2.5	3.7
MM73	0.05	1.1	3.2	0.07	1.9	3.71	1	2.5	3.5
Average	4.09			3.88			3.5		

Represent the highest index of each period.

Table 1: Chitin degradation index of rhizosphere soil bacterial isolates of Mentha and Ocimum plants from Saudi Arabia locations; Jeddah, Mecca and Al-Madinah Al-Munawaraha growing on colloidal chitin agar for 7, 10 and 15 days.

Results showed that chitin degradation index ranged from (2.9 cm to 6.6 cm), (2.7 cm to 4.4 cm) and (2.7 cm to 4.3 cm) after 7, 10 and 15 days respectively.

Among the nine chitinolytic isolates, MM72 isolate showed the maximum chitin degradation activity (6.6 cm) as visualized by zone formation after 7 days, but after 10 and 15 days the AM32 isolate showed the maximum chitin degradation activity (4.4 cm and 4.3 cm), AM31 and JB51 isolates showed the minimum chitin degradation activity (2.9 cm) as visualized by zone formation after 7 days. Among the nine-chitinolytic isolates and AM31 isolate showed the minimum chitin degradation activity (2.7cm) as visualized by zone formation after 10 days and 15 days.

However, the MM33 isolate showed the average chitin degradation activity (4.7 cm) as visualized by zone formation after 7 days. Among the nine-chitinolytic isolates, JM32 and MM33 isolates showed the average chitin degradation activity (3.8 cm) as visualized by zone formation after 10 days. Among the nine-chitinolytic isolates, MM33 isolates showed the average chitin degradation activity (3.5 cm) as visualized by zone formation after 15 days.

The halo zone formation due to the activity of chitinase enzyme bacterial isolates should the best results after 7 days.

Morphological characterization of chitin degrading bacterial isolates

The bacterial strains selected according to its ability to degrade chitin and were morphologically characterized.

Identification of chitin degrading isolates based on 16S rDNA

The nine isolates were identification using DNA sequencing of 16S rDNA.

The samples sequenced in BGI, Hong Kong, China. The sequencing trimmed to eliminated the low quality ends using bioinformatics software: SeqMan pro, DNAstar version 12 by 100 to 250 bases from ends.

The DNA sequences analysed using blastn alignment tools of GenBank and showed that the nine isolates were identified as *Stenotrophomonas maltophilia* with similarity percentages of ranged from 93% to 100%.

The selected isolates submitted into (https://submit.ncbi.nlm.nih.gov/subs/genbank/) under the accession numbers: KT970989 for strain MM7-2 isolated from Mecca -Mentha's rhizospher, KT970990 for strain MM7-3 isolated from Mecca- Mentha's rhizospher, KT970987 for strain JM5-2 isolated from Jeddah- Mentha's rhizospher, KT970988 for strain MM3-3 isolated from Mecca- Mentha's rhizospher, KT970985 for strain JB5-1 isolated from Jeddah-Ocimum's rhizospher, KT970983 for strain AM3-2 isolated from Al-madinah Mentha's rhizospher, KT970984 for strain AM5-1 isolated from Al-Madinah Al-Munawaraha-Mentha's rhizospher, KT970982 for strain AM3-1 isolated from Al-Madinah Al-Munawaraha-Mentha's rhizospher and KT970986 for strain JM3-1 isolated from Jeddah-Mentha's rhizospher.

Phlyogenetic tree analysis of chitinolytic isolates based on 16S rDNA

Figure 3: Neighbor-joining tree showing the phylogenetic position of chitinolytic isolates and their related species based on partial 16S rRNA gene sequences. Strains in red

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box are representing chitinolytic isolates of this study.

The phylogenetic relationship between all chitinolytic isolates analysed in this study versus gene bank strains. According to Figure 3, the chitinolytic isolates divided into three clusters, first cluster contains (KT970989 MM7-2) from Mecca- Mentha's rhizospher, which is the closest relative to (KT970990 MM7-3) from Mecca- Mentha's rhizospher with 0.062% of differences Table S1. At the second cluster it can been see that there are three relationships between chitinolytic isolate as following; (KT970985 JB5-1) from Jeddah Ocimum's rhizospher, which is the most related to (KT970988 MM3-3) from Mecca- Mentha's rhizospher with 0.03% of differences, (KT970983 AM3-2) from Al-Madinah Al-Munawaraha -Mentha's rhizospher and (KT970984 AM5-1) from Al-Madinah Al-Munawaraha -Mentha's rhizospher, which showed 0.008% of differences. However, AM5-1 from Al-Madinah Al-Munawaraha -Mentha's rhizospher is the most closet strain to gene bank strains such as *S. maltophilia* strain SWCH-9, *Stenotrophomonas sp*. RZS7, *S. maltophilia* strain RAT3A, *S. maltophilia* strain LMG 957, *S. maltophilia* strain 17A1, *S. maltophilia* strain S1Y2-aS, *S. maltophilia* strain SaY2-b, *Stenotrophomonas sp*. U18, *S. maltophilia* strain pp5c, *S. maltophilia* strain 1.22, *S. maltophilia* strain173-B, *Stenotrophomonas sp*. IITR87, *S. maltophilia* strain 257-B, *S. maltophilia* strain Dh, *S. maltophilia* strain 257-B, *Stenotrophomonas sp*. PHLE-3, *S. maltophilia* strain PSSB7, *Stenotrophomonas* sp. KD2009-61, *S. maltophilia* strain YHYJ-1, *Pseudomonas hibiscicola* HPG72, *S. maltophilia* strain FF111 and *Stenotrophomonas sp*. USTB-H .The relationship between (KT970982 AM3-1) from Al-Madinah Al-Munawaraha -Mentha's rhizospher and (KT970988 MM3-3) from Mecca- Mentha's rhizospher with 0.012% of differences. In addition, the third cluster indicates only (KT970987 JM5-2) from Jeddah-Mentha's rhizospher.

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