ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology Volume 2 Number 2: 131-140, 2003
ISSN 1682-2978

DNA-fingerprints and Phylogenetic Studies of Some Chitinolytic Actinomycete Isolates

[1]Zaki A. El-Fiky, [2*]Samira R. Mansour, [3]Yahia El-Zawhary and [2]Samar Ismail
[1]Department of Genetics, Fayoum Faculty of Agriculture, Cairo University, Fayoum, Egypt
[2]Department of Botany, Faculty of Science, Suez Canal University, Ismailia, Egypt
[3]Department of Botany, Faculty of Science, Zagazyg University, Zagazyg, Egypt
[*]corresponding author

Abstract: Twenty-one chitinolytic actinomycete-isolates were isolated on chitin agar and were able to degrade chitin as sole carbon source. Total count of chitinolytic active actinomycetes (halo-forming colonies) were significantly lower ($P<0.05$) than those non-active colonies (non -halo-forming colonies). Rizosphere of *Alkanna orientales* plants grown in Saint Catherine area was better habitat for chitinolytic actinomycetes than other rhizospheres of tested plants grown in Suez Canal farm. Using RAPD analysis in an attempt to generate specific markers for chitinolylic actinomycete-isolates, we able to analyzed seven randomly polymorphic DNA primers against the most active chitinolytic actinomycete-isolates. Most of the primers used were useful in identifying unique DNA polymorphisms of all isolates tested. Thirty RAPD-PCR markers were found to be effective to be used as isolate-specific markers. Phylogenetic relationships between the isolates using previous technique (RAPD analysis) revealed that the isolates with higher similarity percent were belong to the same taxonomical group. Using standard techniques of actinomycete identification, morphological and physiological characterization, strengthened these results.

Key words: Chitinolytic actinomycetes - DNA-fingerprint - RAPD analysis - actinomycetes.

Introduction

The existence of actinomycetes has been recognized for over a hundred years. For much of this time they were regarded as an exotic group of organisms with affinities to both bacteria and fungi. However, determination of their fine structure and chemical composition initiated in the 1950s, confirmed their prokaryotic nature. The development of the biology of these filamentous organisms makes such organisms of interest to be studied. They proved to have roles in extreme environments and in many natural processes such as nitrogen fixation, degradation and decomposition of many naturally and synthetic compounds and the control of root pathogens (biological control).

The protection of plant roots against infection by any microbial agents is mainly dependent upon the introduction of hazardous pesticides into environment. Using organisms have capability to control plant pathogens, like those filamentous soil bacteria (actinomycetes), provide an alternative means of reducing the incidence of plant disease without negative aspects of

pesticide application. Interaction between biocontrol agents and plant pathogens have been studied extensively and application of bicontrol agents to protect some commercially important crops is promising (Vasseur ***et al.***, 1990; Chet, 1987 and Boller, 1986). There are two ways by which biocontrol agents can suppers the plant pathogen, production of antibiotics or production of hydrolytic enzymes. Degradation of cell wall of some pathogenic organisms, especially fungi, via hydrolytic enzymes produced by another microorganisms (biocontrol agents) is an important mechanism in biological control. Among hydrolytic enzymes can be produced, as secondary metabolite of biocontrol agents, is chitinase. The role of chitinase in biocontrol of some pathogenic fungi has received increased attention for its effect on fungal pathogens (Morrissey ***et al.***, 1976; Ordentlich ***et al.***, 1988; Oppenheim and Chet, 1992 and Gomes ***et al.***, 2000). Since, the major structural component of cell walls of many plant pathogenic fungi is chitin, which is uniquely absent from higher plants and mammals, it is encouraging to use chitinase-producing organisms as biological control agents. Chitin, an unbranched polysaccharide composed primarily of β-1-4-linked ***N***-acetyl-glucoseamine residues, is hydrolyzed into ***N***-acetyl-glucoseamine (GlcNAc) by chitinase enzyme (Vasseur ***et al.***, 1990 and Gomes ***et al.***, 2000). Chitinases have been detected in a variety of organisms, among them are actinomycetes by which large quantity of these enzymes are produced (Skujins, ***et al.***, 1965 and Sneh, 1981).

For biotechnological importance of chitinolytic actinomycetes genera within the order Actinomycetales as biological control, developing species-specific probes and primers are important in screening bioactive compound produced by specific organism. Genomic fingerprinting assays using randomly amplified polymorphic DNA (RAPD) were proved to be excellent methodologies for differentiating and tracking specific genetic elements within a complex genome (Roberts and Crawford, 2000).

For that purpose, the objectives of our study were to isolate actinomycetes have chitinolytic activity and to generate genetic markers for these isolates through amplification of genomic DNA using oligonucleotide primers (RAPD analysis). Identification and characterization of these isolated depending on their micromorphology, physiology and culture behavior were also carried out. Phylogenetic relationships among the isolates were analyzed.

Materials and Methods

Sample collection

Soil samples were collected from rhizosphere of three different plants, two of them (Tomato and Green pepper plants) were cultivated at two different local sites in the farm of Suez Canal campus and these sites designated S1 and S2 respectively. The third plant (***Alkanna orientales***) was naturally occurred in Saint Catherine area and the site referred as S3. Healthly looking plant were chosen to collect soil rhizosphere. Soil rhizosphere was collected in sterile plastic bags and transferred to the lab for microbial examination. Soil surrounding the plants, at the different sites, were also collected for physical and chemical characterizations.

Isolation technique

Dilution technique was used for isolation of actinomycetes from different collected

rhizosphere soil samples. The samples were diluted (ten-fold) to give final concentrations 10^{-3}, 10^{-4} and 10^{-5}. The best dilution was considered for counting. Isolation of actinomycetes was carried out in accordance with Standard Method (1981) using chitin medium (Willoughby, 1968). Colonies were isolated and purified by Streak Plate Technique.

Determination the most chitinolytic active isolates

To select the most active actinomycetes isolated, the pure culture of each isolated was cultured on the same medium for isolation (chitin agar) by pouring plate technique and the clear zones developed was measured. Three replicated of each isolate were carried out under the same conditions of incubation period (three days) and temperature (30°C). As a result the most active isolates with greater zone of clearness were selected.

Maintenance of isolates

Pure cultures were grown on both chitin medium and Asparagin-glucose agar (Asparagin, 0.5 g; glucose, 10 g; k_2Hp0_4, 0.5; agar, 20 g; distilled H_20, 1000, pH 7.4). The culture were grown for one week and then stored at 5-10°C.

Identification of isolated cultures

For identification and classification of actinomycetes isolates the following criteria were studied: morphological, cultural, physiological and molecular characterizations.

Morphology

The morphology of actinomycete-isolates was examined using slide culture technique (Bergey's Manual of Systematic Bacteriology, 1989). After growth, the slide was taken, left in air to dry, stained with Gram stain and then examined under light microscope.

Cultural characteristics

The growth behavior of actinomycete-isolates were examined at weekly intervals on different media, C1 agar (Nonomura and Ohara, 1969), Asparagin-glucose agar, yeast extract agar (Gordon and Mihm, 1957), glucose peptone agar (Prauser and Falta, 1968), Czapeks agar (Waksman, 1950), Tyrosine agar, Bennett agar (Waksman, 1961) and starch casein agar. The presence of aerial mycelium, the color of aerial and substrate mycelium and formation of soluble pigments were recorded.

Physiological tests

The physiological and biochemical properties were tested for the selected actinomycete-isolates. The tests were carried out in duplicates. Among these are utilization of different carbon source. A variety of carbohydrates were used, mannitol, fructose, inositol and sucrose. Decomposition of casein, tyrosine and hydrolysis of starch were determined by the method of Gordon (1968). Nitrate reduction was determined according to the method described by Gorden (1968) and MacFaddin (1980). Melanin production (Waksman, 1950) was also preformed.

Table 1: Sequences of seven random primers applied for DNA-fingerprints of actinomycete-isolates using RAPD analysis

Primer	Sequence 5 ---------- 3'
A 14	TCTGTGCTGG
A 15	TTCCGAACCC
API	GGTGCGGGAA
AP3	GTAGACCCGT
AP4	AAGAGCCCGT
AP5	AACGCGCAAC
AP6	CCCGTCAGCA

Molecular characterization

DNA extraction

Actinomycete-isolates were propagated in broth medium for ten days at 30°C, for DNA extraction. Extraction was done following the method of EL-Fiky (2003). In this method the actinomycetes-hyphae were harvested by filtration and then washed several times in sterile distilled water. Washed hyphae were gently ground in 120 µl of 50 mM EDTA, followed by 60 µl of lysozyme (10 mg ml^{-1}), after then the mixture was incubated for 30 min at 37°C. The incubated homogenate was then extracted using 600 µl of extraction buffer (20% sucrose, 50 mM Tris-HCl, pH 7.5, 50 mM EDTA) and 60 µl 10% SDS with incubation at 80 °C for 5 min followed by addition of a mixture of phenol: chloroform : isoamyl alcohol (25:24:1). Nucleic acid precipitated with one volume of iso-propanol in presence of 0.3 M ammonium acetate. After drying, micropellets were suspended in 50 µl of TE buffer (10 mM Tris, pH 8, 1 mM EDTA) and stored at -20 °C.

Fingerprinting and RAPD analysis

DNA of the selected isolates was subjected to randomly amplified polymorphic DNA analysis (RAPD) using seven short oligonucleotide primers by which multiple amplification products from loci distribution throughout the genome were resulted. In this analysis, it was prudenting to optimize all parameters of the RAPD analysis for DNA of all the isolates with each of the seven random primers used. In this analysis we used A14 and A15 (Operom random primers) and five primers of AP1 to AP6 (Amersham Pharmacia Biotech RAPD primers). Their sequences were illustrated in Table 1. RAPD reactions were assembled and optimized as described by Williams ***et al.*** (1990), in which the following mixture was prepared, 1 µl dNTPs (0.2 mM), 1.5 µl $MgCl_2$ (1.5 mM), 2.5 µl of 10X reaction buffer, 1.25 µl primer (0.2 µM), 0.5 µl Promega Taq DNA polymerase (2 units), 1µl DNA (100 mg) and sterile distilled water to reach the total volume 25 µl. Negative control was included in which all the above ingredients were added except for DNA (DNA-free). Amplification was carried out in a thermocycler (UNO II, Biometra) programmed for 95°C for 5 min (one cycle) followed by 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min (45 cycles) 72 °C for 5 min (one cycle). Amplification products (7 µl) was mixed with 3 µl loading buffer and separated on 1.5% agarose gel and stained 0.5 µg ml^{-1} ethidium bromide, visualized and photographed under UV light (302 nm) by using Polaroid 667 film. Segregated polymorphismic bands were scored and their patterns were compared with 1Kb DNA ladder marker (Promega Inc.).

Table 2: Selected chemical, physical and microbiological characteristics of three different soil samples collected from soil around plant rhizospheres. Chemical and physical data represent mean of three replicates. Microbiological data represent mean of six- plate counts of actinomycetes colonies on agar plates

Site	designation	pH	Organic matter(%)	Total N(%)	EC (dSm^{-1})	Carbonate (%)
Suez Canal Fram	S1	8.01	0.40	0.08	0.45	5.41
Suez Canal Fram	S2	8.03	0.45	0.09	0.31	5.52
Saint Catherine area	S3	7.61	0.27	0.06	0.13	3.31

Site	designation	Total actinomycetes (starch-casein) ± SD (10^3 g^{-1})	Total actinomycetes (chitin)* ± SD (10^3 g^{-1})	Chitin-clearing cufs** (%)
Suez Canal Fram	S1	44.00±8.98	22.50±5.78	7.00±0.01
Suez Canal Fram	S2	54.33±10.05	24.67±2.89	4.11±0.03
Saint Catherine area	S3	6.40±0.03	1.75±0.15	7.55±0.27

*Total number of actinomycete-colonies g-1 soil that had developed on chitin agar

** percentage of actinomycete-colonies that had chitinolytic activity

Table 3: The occurrence and frequency of chitinolytic genera (halo-forming colonies on chitin-agar) isolated from rhizosphere of following plants: Tomato, Green pepper plants grown in Suez Canal Farm (S I and S2, respectively) and *Alkanna orienta(es* grown in Saint Catherine area (S3).

	Frequency of chitinolytic genera isolated (%)				
Site	*Streptomyces*	*Nocardia*	*Pesudonocardia*	*Nocardiopsis*	*A ctinomadura*
S1	14.30	42.90	28.60	14.20	0.00
S2	66.67	0.00	0.00	0.00	33.33
S3	36.40	54.50	0.00	0.00	9.10

Data obtained from RAPD analysis, for isolates for each random primer, were compared. The similarity indices and the dendogram among the selected isolates were calculated according to Nei and Li (1979) using RAPDistance software package 1.4 (Armstrong ***et al.***, 1994).

Results

Composition of culturable, chitinolytic actinomycetes community

The properties of soil collected from Suez Canal Farm (S1 and S2) were differed considerably from that of Saint Catherine site (S3) with respect to pH and percentage of organic matter (Table 2). The total count of actinomycete-colonies (on both starch casein and chitin media) was significantly higher ($P < 0.05$) in S1 and S2 than in S3 (Table 2).

For each site, the total number of actinomycete-colonies forming units (cfu) on chitin agar were about 5 times lower that those counted on starch-casein agar. Using glucose peptone medium for isolation, the number of colonies developed was of the same order of magnitude as that on starch casein medium (data not shown). Most of colonies developed on chitin agar did not form halos around them (Table 2). In comparing the halo-forming cfu to total count actinomycetes on chitin agar was represented by low percentage (Table 2).

Twenty-one halo-forming actinomycetes have been isolated from all sites selected. Seven isolates from S1, three isolates from S2 and eleven isolates from S3. The data obtained from

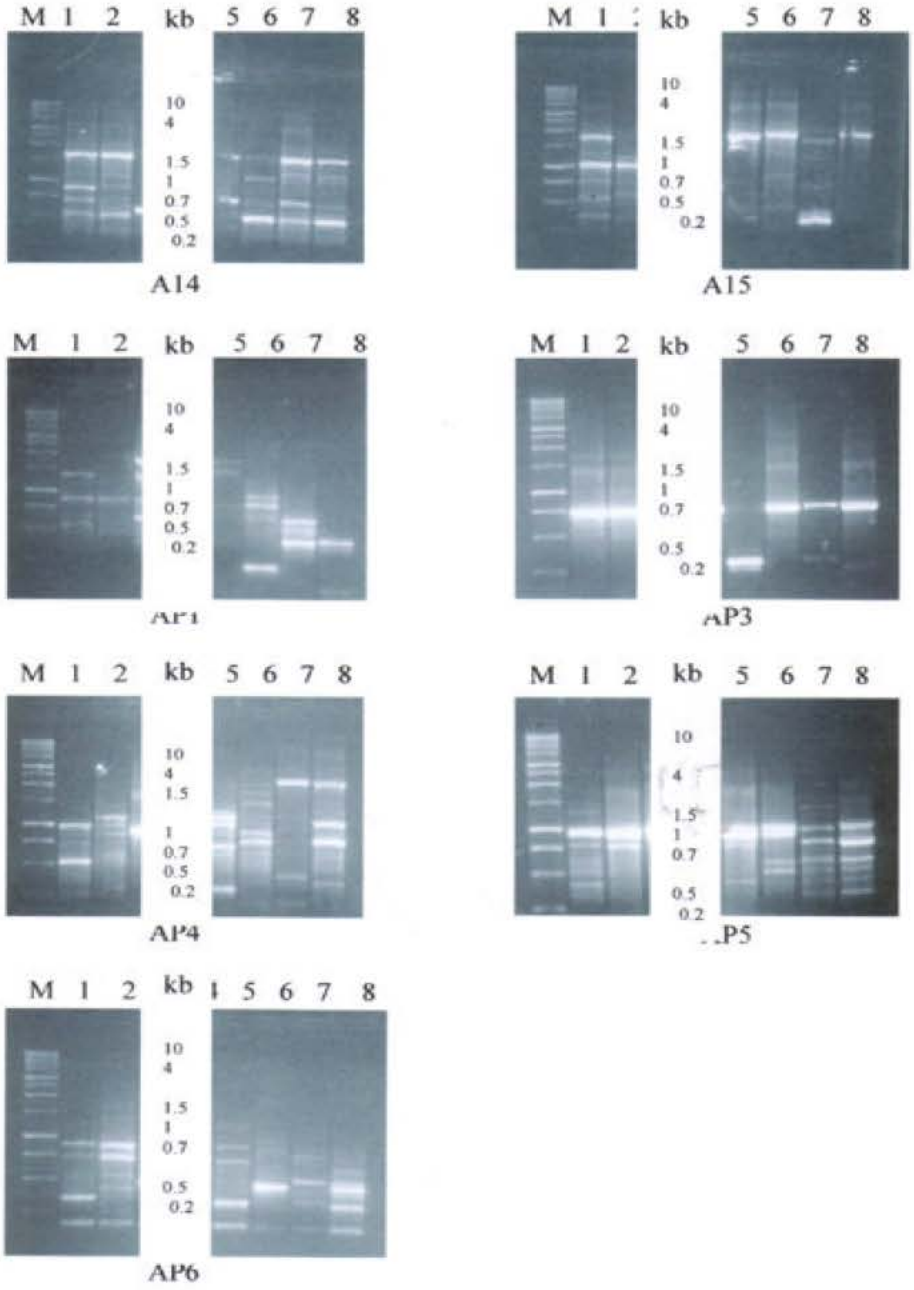

Fig. 1: Image of an ethidium bromide-stained 1.5% agarose gel separation of RAPD reaction products obtained using seven primers, A14, A15, AP1, AP3, AP4, AP5 and AP6 with eight chitinolytic actinomycete isolates. M refers to DNA marker, 1kb ladder (Promega). Amplification were obtained from Isolate 1 (lane 1), Isolate 2 (lane 2), Isolate 3 (lane 3), Isolate 4 (lane 4), Isolate 5 (lane 5), Isolate 6 (lane 6), Isolate 7 (lane 7) and Isolate 8 (lane 8).

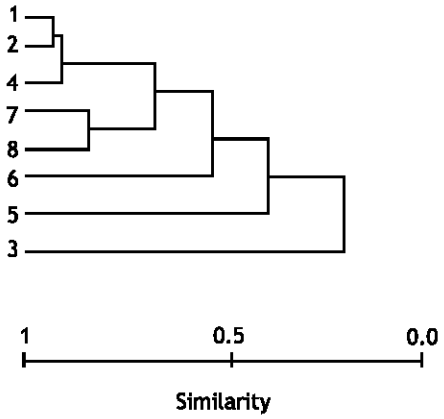

Fig. 2: Dendrogram demonstrating the similarity relationship among eight actinomycetes isolates

determination the chitinolytic active isolates, the most of the halo-forming colonies were from Saint Catherine area (S3). In contrary, S2 (soil collected from rhizosphere of green pepper plants) recorded the lowest percentage of halo-forming colonies. Depending on morphological and physiological characterizations, most of halo-forming isolates, in all sites, appeared to be ***Nocardia*** species, ***Streptomyces***, ***Pesudonocardia***, ***Nocardiopsis*** and ***Actinomadra*** species. The percentage of occurrence of these genera in all sites to total count of halo-forming colonies was 42.86, 33.33 and 9.52%, respectively. The percentage of ***Pesudonocardia***, ***Nocardiopsis*** and ***Actinomadura*** species was the same, which represented by 9.52%. However, the frequency of each identified genus per each site revealed that ***Nocardia*** species recorded the highest frequency for sites S1 and S3 and ***Streptomyces*** species was the highest in site S2 (Table 3).

The most abundant non-halo-forming colonies on chitin-agar consisted of ***Streptomyces*** (in all sites), followed by ***Nocardia*** (all sites), ***Actinomadura*** (S2) and ***Nocardiopsis*** (S3).

RAPD fingerprint profiles

RAPD fingerprint profiles were generated using seven primers on genomic DNA from actinomycete-isolates in order to determine whether these primers might be useful in developing molecular genus-specific markers (in particular, high chitinolytic genus) within the order Actinomycetales. From data obtained, a total number of 96 DNA bands were detected for chosen eight chitinolytic actinomycete-isolates in which 94 of them (represent 97.9%) were useful as polymorphic bands (Fig. 1). Using Primer AP4 and AP1, which generated largest number of polymorphic bands with all isolates used, they might be useful as a marker(s) for number of isolates. Profiles of most interest were those that produced clearly distinguishable major products located between 250 and 3000 bp by primer AP4 (Fig.1). These fragments are promising markers to isolate 1 (***Streptomyces*** spp.) at 250 bp, isolate 8 (***Nocardiopsis*** spp.) at 370 bp, isolate 7 (***Pesudonocardia*** spp.) at 400 bp, isolate 5 (***Nocardia*** spp.) at 670 bp, isolate 4 (***Streptomyces*** spp.), at 1700 bp and isolate 6 (***Nocardia*** spp.) at 3000 bp. For primer AP1, isolates 8, 6, 7, 5 and 4 can be distinguishable at bands with molecular weight between <100 and 4000 bp (Fig. 1).

However using AP3 resulted of the lowest number of polymorphic bands (two bands). These discernible bands were located between 500 and 700 bp (Fig. 1). Using primer AP5, although it produced many bands only one was distinguishable (180 bp) for isolate 4 (***Streptomyces*** spp.).

Generally, thirty RAPD-PCR fragments were found to be useful as specific markers, in which 24 positive specific markers were scored for the presence of unique bands for a given isolate and six negative specific markers were scored for the absence of common bands (Fig. 1).

Phylogenetic relationships among actinomycetes isolates

The genetic relationships among eight actinomycete-isolates are shown in Fig. 2. The dendrogram obtained by RAPDistance software package 1.4, showed that two major group can be distinguished. First group containing three clusters in which the strongest relationship was scored between isolates 2 and 1 (***Streptomyces*** spp.); isolates 2 and 4 (***Streptomyces*** spp. and ***Nocardia*** spp.) and isolates 7 and 8 (***Pesudonocardia*** spp. and ***Nocardiopsis*** spp.) with similarity index 68, 66 and 61%, respectively (Fig. 2). The second group which branched far apart from isolates 1, 2 and 4 was containing three isolates, isolates 6 and 5 (***Nocardia*** spp.) and isolate 3 (***Actinomadura*** spp.). The lowest relationship was scored between isolates 3 and 5 (***Actinomadura*** spp. and ***Nocardia*** spp.) with similarity index 27% (Fig. 2). However, the similarity relationship between isolates 3 and 6 (***Actinomadura*** spp. and ***Nocardia*** spp.) was also not powerful.

Discussion

Most of the filamentous soil bacterial cfu that developed on chitin-agar were apparently not chitinolytic since they did not form a clearing zone. The occurrence of these colonies may be due to the presence of some organic substrates in the agar itself, however, it still in need to examine each single colony for its ability to produced chitinase. Similar results were obtained by Boer ***et al.***, (1998). Chitinolytic cfu actinomycetes was higher in the rhizosphere of ***Alkanna orientales*** plants, collected from Saint Catherine area, although the total actinomycetes count was significantly lower ($P<0.05$) than those collected from plants grown in Suez Canal Farm (Table 2). This observation may be due to different in environmental and soil conditions of both sites. This result is in confirmation with the results obtained by Boer ***et al.***, (1998). In their study, they found that under substrate-limiting conditions the production of chitinase was common and enhanced among chitinolytic soil bacteria.

In our study, the most abundant chitinolytic cfu was found to be belonged to ***Nocardia*** species followed ***Streptomyces*** species. This is in contrast to the results of Boer ***et al.***, (1998) and Ashely ***et al.***, (1998), who observed that most actinomycete-isolates on chitin-agar and chitinolytic active appeared to be ***Streptomyces*** species. Occurrence of ***Pesudonocardia*** and ***Nocardiopsis*** was interesting as chitinolytic actinomycetes, which explored for the first time in this study.

Using randomly amplified polymorphic DNA (RAPD) analysis as a method to generate specific marker(s) by screening unique fragments, we able to detected such fragments with some primers. Therefore, this analysis is generally applicable and powerful for screening for bioactive principles. These results support the results obtained by Roberts and Crawford (2000), Mohamed

et al., (2001) and Mahfouz and Mohamed (2002). However, using this method of analysis as a tool for taxonomic characterization and phylogenetic analysis proved to efficient to some extent. All isolates belong to genus ***Streptomyces*** were grouped together, in two clusters, which is acceptable results depending on their morphological and physiologial characterization. However, genera belong to Nocardiform group (Bergey's Manual of Systematic Bacteriology, 1989) were separated with different similarity indices (Fig. 2). This result is in need for more investigation to study the sequences of the fragments produced and their homology for these isolates. In conclusion using RAPD analysis is recommended to be used in identifying unique DNA polymorphism of target organisms especially those with hydrolytic activity and these can be used as markers.

References

Armstrong, J.S., A.J. Gibbs, R. Peakall and G. Weiller, 1994. http://life.anu.edu.au/molecular/software/rapd.html.

Ashely, A.B., J.C. Hubbard and L. Lui, 1998. Effects of chitin and chitosan on the incidence and severity of Fusarium yellows of celery. Plant disease, 82: 322-328.

Bergey's Manual of Systematic Bacteriology, 1989. Williams, S.T., M.E. Sharpe and J.G. Holt, (Eds.), volume 4. Williams and Wilkins Co., Baltimore.

Boer, W.D., P.J.A. Klein Gunnewiek, P. Lafeber, J.D. Janse, B.E. Spit and J.W. Woldendorp, 1998. Anti-fungal properties of chitinolytic dune soil Bacteria. Soil Biol. Biochem., 30: 193-203.

Boller, T., 1986. Chitinase: a defense of higher plants against pathogens. In Muzzarelli, Jeuniaux, C. and Gooday, C.W., (Eds.), Chitin in Nature and Technology. New York. Plenum Press, pp: 223-231.

Chet, I., 1987. Trichoderma-application, mode of action and potential as a biocontrol agent of soil-borne plant pathogenic fungi. In: Chet (Ed.). Innovative Approaches to Plant Disease Control. New York: John Wiley and Sons, pp: 137-160.

El-Fiky, ZA., 2003. A simple and rapid method for a mini preparation of high molecular weight DNA from certain acarine, bacteria and the soybean plant. Ins. Sci. and its Appl., 23: 51-57.

Gomes, R.C., L.T.A.S. Semedo, R.M.A. Soares, C.S. Alviano, L.F. Linhares and R.R.R. Coelho, 2000. Chitinolytic activity of actinomycetes from a cerrado soil and their potential in biocontrol. Letters in applied Microbiol., 30: 146-150.

Gordon, R.E., 1968. The taxonomy of soil bacteria. In: Gray, Parkinson (Eds.), The Ecology of Soil Bacteria. Liverpool University Press, Liverpool, England, pp: 293-321.

Gordon, R.E. and J.M. Mihm, 1959. A comparison of ***Nocardia asteroides*** and ***Nocardia brasiliensis*** J.Gen. Microbiol., 20:129-135.

MacFaddin, J.F., 1980. Biochemical tests for identification of Medical Bacteria. 2nd Ed. Williams and Wilkins, Baltimore.

Mohamed, S.H., H.I. Abdel-Fattah, S.M. Seliem and M.S. Sharaf, 2001. Identification and molecular studies on some hallotolerant actinomyctes isolated from Sinai sandy soil. Arab J. Biotech., 4: 179-196.

Mahfouz, H.T. and S.H. Mohamed, 2002. Physiological, antagonistic and fingerprinting studies on some halotolerant ***Streptomyces*** strains. Arab. J. Biotech., 5: 103-120.

Morrissey, R.F., E.P. Dugan and J.S. Koths, 1976. Chitinase production by an ***Arthrobacter sp.*** lysing cells of ***Fusarium roseum***. Soil Biol. Biochem., 8: 23-28.

Nei, M. and W.H. Li, 1979. Mathematic model for studing genetic variations in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA., 76: 5269-5273.

Nonomura, H. and Y. Ohara, 1969. Distribution of actinomycetes in soil. VII. A culture method effective for both preferential isolation and enumeration of Microbispora ans Streptosporangium strains in soil (Part 2). Classification of isolates. J. Ferment.Technol., 47: 701-709.

Ordentlich, A., Y. Elad and I. Chet, 1988. The role of chitinase of ***Serratia marcescens*** in biocontrol of ***Sclerotium rolfsii***. Phytopathology, 78: 84-87.

Oppenheim, A.B. and I. Chet, 1992. Cloned chitinase in fungal pathogen control strategies. Trends in Biotechnology, 10: 392-394.

Roberts, M.A. and D.L. Crawford, 2000. Use of randomly amplified polymorphic DNA as a means of developing genus- and strain-specific ***Streptomyces*** DNA props. Applied and Environ. Microbiol. 66: 2555-2564.

Skujins, J.J., H.J. Potgieter and M. Alexander, 1965. Dissolution of fungal cell walls by a streptomycete chitinase and β-(1 → 3) glucanase. Arch. Biochem. and Biophy., 111: 358-364.

Sneh, B., 1981. Use of rhizosphere chitinolytic bacteria for biological control. Phytopathologische Zeitschrift, 100: 251-256.

Standard Method for the Examination of Water and Wastewater, 1981. 15 th Ed. New York American Puplic Health Association.

Vasseur, V., F. Arigoni and H. Anderson, G. Defago, G. Bompeix and J.M. Seng, 1990. Isolation and characterization of ***Aphanocladium album*** chitinase over producing mutants. J. General Microbiol., 136: 2561-2567.

Waksman, S.A., 1950. The Actinomycetes-their nature, occurrence, activities and importance. Chronic Botanica Co., Waltham, Massachusetts.

Waksman, S.A., 1961. The Actinomycetes. Classification, identification and descriptions of genera and species, Vol. 2, The Williams and Wilkins Co., Baltimore, pp: 1-363.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. 18: 6531-6535.

Willoughby, L.G., 1968. Aquatic ***Actinomycetales*** with particular reference to the ***Actinoplanaceae***. Veroeff. Inst.Meersforsch. Bremerhaven, 3:19-26.