Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 6, Issue 2, 2013 ISSN - 0974-2441

Research Article

ISOLATION AND CHARACTERIZATION OF AN ENDOPHYTIC BACTERIUM FROM *BRASSICA OLERACEA* WITH POTENTIAL ENZYME AND ANTIBACTERIAL ACTIVITY

SWETHA SUNKAR* AND C. VALLI NACHIYAR

Department of Biotechnology, Sathyabama University, Chennai, India. Email: swethauk78@gmail.com

Received: 16 February 2013, Revised and Accepted: 11 March 2013

ABSTRACT

Endophytes includes a suite of microorganisms that grow intra-and/or intercelullarly in the tissues of higher plants without causing over symptoms on the plants in which they live and have proven to be rich sources of bioactive natural products. In the present study, an endophytic bacterium has been isolated from *Brassica oleracea* (Cabbage) and was identified as *Pseudomonas aeruginosa* by various biochemical tests and 16s rRNA analysis. Two aspects of the endophyte was studied namely enzyme quantification and antimicrobial activity. The enzyme activity was found to be 2 IU/ml, 1.5 IU/ml and 1.9 IU/ml respectively for amylase, protease and cellulase. The endophyte was showing significant antimicrobial activity against *Klebsiella pneumoniae* (20 mm), *Staphylococcus aureus* (25 mm), *Escherichia coli* (13 mm) and *Salmonella typhi* (12 mm).

Keywords: Endophyte; *Brassica oleracea*; *Pseudomonas aeruginosa*; 16s rRNA; Enzymes; Antibacterial activity

INTRODUCTION

Since time immemorial there has been a continuous involvement and interaction between the living organisms including plants and animals with diverse microbes as a part of their natural survival. Certain bacteria and fungi termed endophytes are capable of residing within the plant tissues without causing any harm and could also establish a mutualistic association [1-3]. Plants constitute vast and diverse niches for these endophytic organisms. It is worth mentioning that each plant species is a host to a number of bacteria. Bacteria living inside plant tissues form associations ranging from pathogenic to symbiotic. Beneficial relationships include symbiosis, diazotrophic endophytes that supply the plant with fixed nitrogen and other endophytic associations that promote plant growth by producing phytohormones, volatiles, defense compounds, and enzymes [4, 5]. Endophytic bacteria have been isolated from a large diversity of plants as reviewed by Sturz and associates in 2000[6].

Endophytes are sheltered from environmental stresses and microbial competition by the host plant and they seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species [7]. Some endophytic bacteria exert several beneficial effects on host plants, such as stimulation of plant growth [8], nitrogen fixation [9-11] and induction of resistance to plant pathogens [12-15].

It has been earlier reported that some of the endophytic bacteria belonged to members of diverse genera namely *Pseudomonas*, *Burkholderia* and *Bacillus* [16]. These genera are well-known for production of their secondary metabolites namely antibiotics, anticancer agent like Taxol [17], antiviral compound such as Cytonic acid A [18] and Cytonic acid B, insecticidal agent like Oocydin[19] and some immune suppressant agents. Their role in phytoremediation has also been studied in certain strain of Methylbacterium which was isolated from hybrid poplar trees (*Populus deltoids x nigra*) that was able to biodegrade many nitro-aromatic compounds such as 2, 4, 6- trinitrotoluene [20]. It was found that bacteria degrading recalcitrant compounds are more abundant among endophytic populations than in the rhizosphere of the plants in contaminated sites [21], which could mean that endophytes play a role in metabolizing these substances.

Microbial resistance to the existing drugs used in the treatment of many infectious diseases is increasing and new resistance problems are emerging, further complicating and rendering treatment of critical infectious illness more difficult [22] and this turned the search for new antimicrobial agents an important strategy for alternative therapies useful in the handling of difficult infections [23]. Natural sources continue to be an important supply of new pharmaceutical products [24]. Metabolites isolated from the endophytes are good sources of novel secondary metabolic products having diverse structural groups and showing antibacterial, antifungal, anticancer, antiviral, antioxidant, insecticide, antidiabetic and immunosuppressive activities [25-26].

The importance of the microorganisms in enzyme production has been an area of constant research due to their high production capability, low cost and susceptibility to genetic manipulation. In reality, the enzymes of microbial origin have high biotechnological interest such as in the processing of foods, manufacturing of detergents, textiles, pharmaceutical products, medical therapy and in molecular biology [27-28]. This necessitated the identification of microorganisms that produce enzymes for specific substrate, with different requirements in temperature, pH and presence of different ions, for different production processes [27]. The endophytic microorganisms occupy a relatively unexplored site with respect to enzyme production and so they can represent a new source in obtaining more enzymes with different potentialities.

The present investigation developed as a curiosity to uncover the presence of endophytes in the edible food Cabbage. *Brassica oleracea* (Cabbage) is a cool season crop with a high cold tolerance, suitable for both fresh and processed products. The only edible part of the plant is the leafy head, more precisely, the spherical cluster of immature leaves which is an excellent source of vitamin C. It has significant amounts of glutamine, an amino acid that has anti-inflammatory properties. Cabbage is a source of indole-3-carbinol, a chemical which boosts DNA repair in cells and appears to block the growth of cancer cells. It is surprising to find that the leaves of *Brassica oleracea* housed an endophytic bacteria that was identified as *Pseudomonas aeruginosa* with potential antibacterial activity and also capable of producing certain key enzymes namely amylase, protease and cellulase.

MATERIALS AND METHODS

Isolation and identification of endophytic bacteria

Healthy edible leaves of *Brassica oleracea* were collected and cleaned under running tap water to remove debris and then air dried. Four segments of 1 cm length were cut and surface sterilization was carried out by submerging them in 75% ethanol for 2 mins. The explants were further sterilized sequentially in 5.3% sodium hypochlorite (NaOCl) solution for 5 min and 75% ethanol for 0.5 min and placed horizontally on separate Petri dishes containing Nutrient Agar. After incubation at 32°C for three days, individual bacteria were collected and placed onto nutrient agar and incubated for 3 days and checked for culture purity. Eventually, pure cultures were transferred to nutrient agar slant tubes and subcultured

regularly [30]. The isolated endophytic bacterium was identified based on morphological characterization and various biochemical tests. The identification at molecular level was carried out by 16s rRNA analysis [31].

Culture conditions

Endophytic bacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of sterilized nutrient broth and incubated for 48 h at 31 ± 1ºC in shaker at 125 cycles/min. After the incubation period, culture media were centrifuged at 10,000 g for 15 min and the supernatant was collected by filtration. This is used as the starting material for enzyme and antimicrobial activity assay [32].

Antibacterial Activity

The antibacterial activity has been studied against the test organism that include *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Salmonella typhi* ATCC 6539 and *Klebsiella pneumoniae* NCIM 2883 by agar well diffusion method [33]. The bacterial test organisms were grown in nutrient broth for 24 h. 1 ml nutrient broth culture of each bacterial organism was used to prepare bacterial lawns on nutrient agar. Agar wells were prepared with the help of a sterilized stainless steel cork borer. The wells were loaded with 100µl of the supernatant obtained after centrifugation and filtration and incubated overnight to observe the zones of inhibition along with standard antibiotic streptomycin, amoxicillin and ofloxacin.

Enzyme Activity

The activity of the enzymes namely amylase, protease and cellulase were screened by inoculating the endophytic bacterium on a growth media supplemented with starch, casein and CMC as substrates respectively. The activity was observed by the formation of clear zones around the colonies.

Amylase Assay

Amylase assay was performed using Dinitrosalicyclic acid method. The reaction mixture contained 1 ml of standard starch solution (1% soluble starch in 50mM phosphate buffer, pH 6.9) and incubated for 1 hr at 45° C. The reaction was stopped by adding 3 ml of dinitrosalicyclic acid and boiled for 5 min and cooled. Absorbance of the resulting solution was determined at 550 nm. The amount of sugars produced was read from standard curve using glucose as standard. Enzyme activity was expressed in units where 1 unit/ml is the amount of enzyme which releases 1 µl mole of glucose under the assay condition [34].

Protease assay

Protease production was assayed by a modified method of Kunitz (1947). Samples containing 400 µl of 0.5 % (w/v) casein in 50 mM Tris –HCl buffer, pH 10, with 100 µl enzyme solution were incubated in a water bath at 50 °C for 20 min. The enzyme reaction was terminated by addition of 500 µl of 10 % (w/v) trichloroacetic acid and was kept at room temperature for 10 min. The reaction mixture was centrifuged at 10,000 g for 10 min at 4 °C and the absorbance was measured against a blank at 280 nm. One unit of proteases was defined as the amount of the enzyme releasing an equivalent of one µmol of tyrosine per minute under the defined assay conditions [35].

Cellulase Assay

The reaction mixture for the cellulase assay contained 1 ml of culture supernatants and CMC in 1 ml of 0.1 M sodium phosphate buffer, pH 6.5. After incubation at 60°C for 10 min the reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent. The tubes were placed in a boiling water bath for 50 min and then cooled to room temperature and filtered. The optical densities of the filtrates were measured at 570 nm and converted to glucose equivalents [36].

RESULTS AND DISCUSSION

One of the diverse groups of microbes in the myriad ecosystems is the endophytes that exhibit wide chemical diversity because of the constant chemical innovations that occur within them for their survival. Endophytes are a storehouse of various bioactive metabolites that is playing a significant role in medical industry as the importance of natural products is on the rise. The potential of endophytes can be further harnessed in biotechnological applications as microbes by themselves play a crucial role in the industry. Hence the isolation of endophytes from various sources is gaining momentum so as to tap the innumerable benefits offered by them in various spheres of life.

Isolation of Endophytes

The growth of one endophytic bacterium named as isolate CBGP was observed from the cut edge of the surface sterilized leaves of *Brassica oleracea* after 24 hrs on the nutrient agar plates (Fig 1). Considerable growth was observed after 24 hrs and this strain was isolated, grown and subsequently pure cultures were maintained on nutrient agar slants as well as in 10% glycerol at 4°C. The preliminary identification of the bacterial isolate was done based on morphological characteristics and various biochemical tests (Table 1).

Table 1: Biochemical characteristics of the endophytic bacterium.

S.No	Test	Result
1	Grams staining	-
2	Indole	-
3	Methyl red	-
4	Voger proskaure	-
5	Citrate	+
6	Urease	-
7	Motility	+
8	Oxidase	+
9	Catalase	+

The bacterium was observed to be gram negative and rod shaped without spore formation. Based on the results obtained in table 1 the isolate was identified as *Pseudomonas* sp. It has been earlier demonstrated that endophytes were housed by the different species of coffee plants [37] and soyabean [15]. This result is in accordance with the results of Mendes who reported the occurrence of *Pseudomonas* as an endophyte in sugar cane [29]. Another study by Shin [38] revealed the presence of *Pseudomonas* sp. as endophytes in the roots of sand dune plants that constituted to 49% of the total endophytes.

Further identification at the species level was carried out by 16s rRNA analysis. 16S ribosomal RNA, a component of the 30S small subunit of prokaryotic ribosomes is approximately 1.5kb in length. The genes coding for it referred to as 16S rDNA are used in reconstructing phylogenies which was pioneered by Carl Woese [39]. The 16SrRNA gene is used for phylogenetic studies [40] as it is highly conserved between different species of bacteria and archaea [41]. 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification. Universal PCR primers are used to amplify the 16SrRNA gene providing the phylogenetic information, the most common universal primer pair was devised by Weisburg et al. [40].

The endophytic bacterial DNA was isolated and the 16S rDNA sequence was amplified using the universal primers and sequenced. The 16S rDNA sequence thus obtained was compared with the non-redundant BLAST database in order to acquire the sequences that displayed maximum similarity (Table 2). All the sequences reported by BLAST program revealed that the 16S rDNA sequence of the endophytic bacterial species designated as CBGP showed a very high percentage of similarity (99%) with the sequences of *Pseudomonas aeruginosa*, with a reasonably high score and e-value being zero.

Table 2: Sequences showing maximum similarity to the endophytic rDNA sequence

Accession	Description	Total score	E - value	Max identity
EF511519.1	Uncultured bacterium clone P5D15-397 16S ribosomal RNA gene	2603	0.0	99%
HQ844508.1	Pseudomonas aeruginosa strain XZPG11 16S ribosomal RNA gene	2601	0.0	99%
HQ844488.1	Pseudomonas aeruginosa strain NYHS11 16S ribosomal RNA gene	2601	0.0	99%
HM030992.1	Pseudomonas aeruginosa strain zgkd2 16S ribosomal RNA gene	2601	0.0	99%
HQ143612.1	Pseudomonas aeruginosa strain RHH13 16S ribosomal RNA gene	2601	0.0	99%
HM752579.1	Pseudomonas aeruginosa strain LLJQ-1 16S ribosomal RNA gene	2601	0.0	99%
FJ972530.1	Pseudomonas aeruginosa NO2 16S ribosomal RNA gene	2601	0.0	99%
FJ972527.1	Pseudomonas aeruginosa CJM 16S ribosomal RNA gene	2601	0.0	99%

The phylogenetic relationship between the homologous sequences obtained by BLAST program was constructed using CLUSTALW employing the neighbor-joining algorithm. This relationship is important to identify the species related to the endophyte.

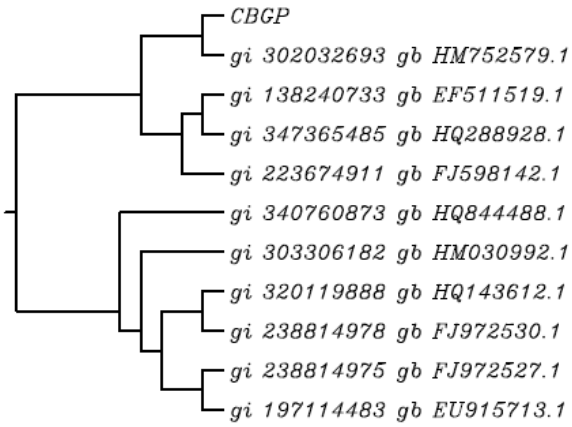

Fig 2:Phylogenetic tree depicting the relation between the 16s rDNA sequence of the endophytic bacterium with its possible homologous sequences.

The evolutionary relationship is depicted in the form of a dendrogram (Fig 2) that shows a clear rooted evolution. All the sequences are shown to be derived from a common ancestor who later diverged into two different clusters that grouped the various strains of *Pseudomonas aeruginosa.* Based on the results obtained and correlating with the results from morphological and biochemical tests it has been identified that the endophytic bacterium isolated is *Pseudomonas aeruginosa.*

Enzyme Activity

The importance of enzymes as biocatalysts and their invariable use in industry for a variety of applications ranging from food industry to leather industry makes their synthesis on a large scale indispensable. Initial sources of enzymes were animals and plants that were followed by a search for better, less expensive and more readily available sources of such enzymes. This resulted in the use of microbes that is a repository of different kinds of enzymes which can be hydrolyzing, oxidizing or reducing, and metabolic in nature. Hence, it is imperative to screen the microorganisms for the activity of specific enzymes.

The endophytic bacterium *Pseudomonas aeruginosa* was screened for the presence of amylase, protease and cellulase using the specific substrates starch, casein and CMC respectively. The positive results are shown by the *Pseudomonas aeruginosa* in terms of zone of clearance around the bacterial colony. This is followed by the quantification of enzyme production by using the standard assay procedures mentioned above. It has been observed that the endophytic bacterium *Pseudomonas aeruginosa* was showing considerable enzyme activity for amylase (2 U/ml), protease (1.5 U/ml) and cellulase (1.9 U/ml). It is observed that this endophytic bacterium displayed maximum amylase activity followed by cellulase and protease. It was earlier reported by Liu et al that marine *Pseudomonas* sp. was showing significant alpha amylase production [42].

Antibacterial Activity

The discovery of novel antimicrobial metabolites from endophytes is an important alternative to overcome the increasing levels of drug resistance by plant and human pathogens. [43]. The production of bioactive substances by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to better adapt to plant host and carry out some functions such as protection from pathogens, insects, and grazing animals [44]. Endophytes are chemical synthesizer inside plants [45], in other words, they play a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms [44].

The bactericidal activity of culture supernatants of the endophytic bacterium *Pseudomonas aeruginosa* was studied against a few test organisms namely *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae* and *Salmonella typhi.*

Table 3: Zones of inhibitions produced by the endophytic *Pseudomonas* and standard antibiotics

Test Organism	Zones of Inhibition (mm)	Amoxicillin	Streptomycin	Ofloxacin
E.coli	13	14	15	13
Klebsilla pneumoniae	20	14	10	13
Staphylococcus aureus	25	5	12	15
Salmonella typhi	12	15	12	15

After the incubation time, clear zones were observed against all the test organisms and were recorded in millimeters (Table 3). These results corroborate with the results obtained by Krid in 2010 [46] where in the endophytic *Pseudomonas* sp. was displaying substantial antagonistic activity in vitro. The antimicrobial compounds that have been characterized previously from *P. aeruginosa* include a group of peptides known as pyocins and an assortment of small heterocyclic compounds including the phenazines, quinolines and phenylpyrroles. These compounds destroy a range of other microorganisms through mechanisms ranging from DNA damage [47] to cell depolarization [48]. In the present study, the culture supernatant of *Pseudomonas aeruginosa* was exhibiting significant activity against *K. pneumoniae* and *S. aureus* in comparision with the standard antibiotics. Hence this study reinforces the fact that if the appropriate conditions are provided, this endophytic bacterial species could house a lot of antimicrobial compounds that can be further isolated and characterized.

CONCLUSION

Endophytes have proven to be rich sources of novel natural compounds with a wide-spectrum of biological activities and a high level of structural diversity. The use of endophytes as biocatalysts in the biotransformation process of natural products assumes greater importance. An endophytic bacterium was isolated from the edible portions of *Brassica oleracea*. Preliminary identification from the various biochemical assays revealed that the bacteria is gram

negative rod shaped belonging to the genera *Pseudomonas* and this was further characterised by 16s rRNA analysis from which it has been identified that the endophyte was *Pseudomonas aeruginosa*. This endophyte possessed was explored for its antibacterial activity and its ability to produce enzymes. This study revealed that this endophyte was showing significant antibacterial activity in comparison with the standard antibiotics. A detailed study on the isolation of antibacterial compounds is progressing. Similarly this bacterium also owns the inherent ability to produce some of the important enzymes like amylase, protease and cellulase. Optimizing the parameters would further enhance the production on a much larger scale opening another area of research.

REFERENCES

1. Azevedo JL, Maccheroni W Jr., Pereira JO, Araújo WL. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *Elect. J. Biotech*. http:// www.ejbiotechnology.info/content/vol3/issuel/full/4/inde x.html
2. Hallmann JM, Quadt-Hallmann A, Mahaffee WF, Kloepper JW. Bacterial endophytes in agricultural crops, *Can. J. Microbiol*, 1997, 43, 895-914.
3. Perotti R. On the limits of biological enquiry in soil science. *Proc. Int. Soc. Soil Sci*. 1926, 2,146-161.
4. Hooper LV, Wong MH., Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. *Science*, 2001, 291, 881-884.
5. De Matos Nogueira E, Vinagre F, Masuda HP, Vargas C, de Pádua VLM, da Silva FR, dos Santos RV, Baldani JI, Gomes Ferreira PC, Hemerley. Expression of sugarcane genes induced by inoculation with *Gluconacetobacter diazotrophicus* and *Herbaspirillum rubrisubalbicans. Genet. Mol. Biol*., 2001, 24,199-206.
6. Sturz AV, Christie BR, Nowak J. Bacterial endophytes: Potential role in developing sustainable systems of crop production. *Crit. Rev. Plant Sci*. 2000, 19,1-30.
7. Kobayashi DY, Palumbo JD. Bacterial endophytes and their effects on plants and uses in agriculture. In: Bacon CW and White JF (Eds.) Microbial endophytes. Marcel Dekker, Inc. NewYork, pp. 199-233.
8. Sturz AV, Christie BR, Matheson BG. Nowak J. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth, *Biol. Fertil. Soils*, 1997, 25, 13 – 19.
9. Kirchhorf G, Reis VM, Baldani JI, Eckert B, Döbereiner J, Hartmann A. Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in gramineous energy plants. *Plant Soil*, 1997, 194, 45-55.
10. Stoltzfus JR, So R, Malarvithi PP, Ladha JK, de Bruijn F J. Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen, *Plant Soil*, 1997, 194, 25-36.
11. Hurek TB, Reinhold-Hurek M, van Montagu, Kellenberger E. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *J. Bacteriol*, 1994, 176, 1913-1923.
12. Chen C, Bauske EM, Mussan G, Rodriguez-Kabana R, Kloepper JW. Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria . *Biol. Control*, 1995, 5, 83-91.
13. Liu L, Kloepper JW, Tuzun S. Induction of systemic resistance in cucumber against *Fusarium* wilt by plant growth-promoting rhizobacteria. *PhytoPath*, 1995, 5, 695-698.
14. Sturz AV, Matheson BG. Populations of endophytic bacteria which influence host-resistance to Erwinia induced bacterial soft rot in potato tubers. *Plant Soil*, 1996, 184, 265-271.
15. Pham Quang Hung, Annapurna K. Isolation and Characterization Of Endophytic Bacteria In Soybean (Glycine Sp.) *Omonrice*, 2004, 12, 92-101
16. Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M, Van der Lelie D. Endophytic bacteria and their potential applications. *Crit Rev Plant Sci*., 2002, 21, 583–606.
17. Strobel GA, Stierle A, Stierle D, Hess WM. Taxomyces a proposed new taxon for a bulbilli ferrous hyphomycete associated with Pacific yew. *Mycotaxon*., 1993, 47, 71–78 .
18. Guo B, Dai J R ,Ng S, Huang Y, Leong C ,Ong W and Carte B K. Cytonic acids A and B: Novel tridepside inhibitors of hCMV protease from the endophytic fungus *Cytonaema* species. *J Nat Prod*., 2000, 63,602-604.
19. Strobel G, Daisy B, Castillo U, Harper J. Natural products from endophytic microorganisms. *J Nat Prod*. 2004, 67, 257–268.
20. Van A B, Peres C, Doty S, Yoon J, Schnoor J. *Methylobacterium populi* sp. nov., a novel aerobic, pink pigmented, facultatively methylotrophic, methane-utilizing bacterium isolated from poplar trees (*Populus deltoides x nigraDN34*). *Evol Microbio*., 2004, 54, 1191–1196.
21. Siciliano S, Fortin N, Himoc N. Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. *Appl Environ Microbiol*., 2001, 67, 2469–2475.
22. Henrique Pereira Ramos, Glaucia Hollander Braun, Mônica Tallarico Pupo and Suraia Said. Antimicrobial Activity from Endophytic Fungi Arthrinium state of *Apiospora montagnei Sacc*. and *Papulaspora immerse*. *Brazilian Arch Biol Tech*, 2010, 53(3), 629-632.
23. Levy SB. Antibiotic resistance-the problem intensifies. *Adv. Drug. Deliv. Rev*., 2005, 57, 1446-1450.
24. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. *J. Nat.Prod*., 2007, 70, 461-477.
25. Strobel GA, Miller RV, Miller C, Condron M, Teplow DB, Hess WM. Cryptocandin, a potent antimycotic from the endophytic fungus *Cryptosporiopsis cf. quercina*. *Microbiol*, 1999, 145, 1919-1926.
26. Lee J, Lobkovsky E, Pliam NB, Strobel GA, Clardy J. Subglutinols A and B: immunosuppressive compounds from the endophytic fungus *Fusarium subglutinans*. *J. Org. Chem*, 1995, 60, 7076-7077.
27. Falch EA. Industrial enzymes - developments in production and application. *Biotechnol Adv*, 1991, 9, 643-658.
28. Rao MB, Tanksale AM, Ghatge M S, Deshpand VV. Molecular and Biotechnological Aspects of Microbial Proteases. *Microbiol Mol Biol Rev*, 1998, 62, 597-635.
29. Rodrigo Mendes, Aline A. Pizzirani-Kleiner, Welington L. Araujo, Jos M Raaijmakers. Diversity of Cultivated Endophytic Bacteria from Sugarcane: Genetic and Biochemical Characterization of Burkholderia cepacia Complex Isolates. *Appl. Environ. Microbiol*. 2007, 73 , 22 7259-7267.
30. Rong-lin He, Guo-ping Wang, Xiao-Hong Liu, Chu-long Zhang and Fu-cheng Lin. Antagonistic bioactivity of an endophytic bacterium isolated from Epimedium brevicornu Maxim African Journal of iotechnology 2009, 8 (2), 191-195,
31. Madhva Charyulu E, Sekaran G, Susheeela Rajakumar G, Armugam Gnanamani. Antimicrobial activity of secondary metabolite from marine isolate *Pseudomonas* sp. against gram positive and negative bacteria including MRSA. *Indian J exp biol*, 2009, 47, 964-968.
32. Amanda S. Motta, Florencia Cladera-Olivera, Adriano Brandelli. Screening for antimicrobial activity among bacteria isolated from the Amazon basin. *Brazilian J Microbiol.* 2004, 35, 307-310.
33. Schillinger U, Lucke FK. Antibacterial activity of *Lactobacillus*, isolated from meat. *Appl. Environ. Microbiol*, 1989, 55, 1901-1906.
34. Sasmita Mishra and Niranjan Behera. Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes. *AfricanJj Biotech*, 2008, 7 (18),3326-3331
35. Abdelnasser SS, Ibrahim Nefisa MA, EI-Shayeb, Sohair S Mabrouk. Isolation and Identification of Alkaline Protease Producing Alkaliphilic Bacteria from an Egyptian Soda Lake. *J Appl Scis Res*. 2007, 3(11), 1363-1368.
36. Lee BH, Blackburn TH. Cellulase Production by a *Thermophilic Clostridium* Species. *Appl Microbiol*. 1975, 30(3), 346-353

37. Humberto Franco Shiomi, Harllen Sandro Alves Silva, Itamar Soares de Melo, Flávia Vieira Nunes, Wagner Bettiol. Bioprospecting endophytic bacteria for biological control of coffee leaf rust. *Sci. Agric*. (Piracicaba, Braz.), 2006, 63(1), 32-39.
38. Shin DS, Park MS, Jung S, Lee MS, Lee KH, Bae KS, Kim SB. Plant growth-promoting potential of endophytic bacteria isolated from roots of coastal sand dune plants. *J Microbiol Biotechnol*. 2007, 17(8),1361-1368.
39. Woese CR. Bacterial evolution. *Microbiol Rev*. 1987, 57, 221-271.
40. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study, *J Bacteriol*. January; 1991, 173(2), 697-703
41. Coenye T, Vandamme P. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol. Lett*. 2003, 228 (1), 45–49.
42. Jianguo Liu, Zhiqiang Zhang, Hu Zhu, Hongyue Dang, Jianren Lu, Zhanfeng Cui. Isolation and characterization of α-amylase from marine *Pseudomonas* sp. K6-28-040 *African J Biotech*, 2011, 10(14), 2733-2740.
43. Liu X, Dong M, Chen X, Jiang M, Lv X, Zhou J. Antimicrobial activity of an endophytic *Xylaria* sp.YX-28 and identification of its antimicrobial compound 7-amino-4-methylcoumarin, *Applied Microbiology and Biotechnology*, 2008, 78, 241–247.
44. Owen NL, Hundley N. Endophytes—the chemical synthesizers inside plants, *Science Progress*, 2004, 87, 79–99.
45. Strobel GA. Endophytes as sources of bioactive products, *Microbes and Infection*, 2003, 5(6), 535–544.
46. Krid S, Rhouma A, Mogou I, Quesada JM, Nesme X, Gargouri A. *Pseudomonas Savastanoi* Endophytic Bacteria In Olive Tree Knots And Antagonistic Potential Of Strains Of *Pseudomonas fluorescens* And *Bacillus Subtilis Journal Of Plant Pathology*, 2010, 92 (2), 335-341
47. Kerr J. Phenazine pigments, antibiotics and virulence factors. *The Infectious Disease Review—microbes of man, animals and the environment*, 2000, 2(4), 184-194.
48. Michel-Briand Y, Baysee C. The pyocins of *Pseudomonas aeruginosa*. *Biochimie*, 2002, 84, 499-510.