ALOE VERA: CULTIVABLE BACTERIAL ENDOPHYTES IN PRODUCTION OF POLYKETIDES AND NON RIBOSOMAL PEPTIDES ASSOCIATED WITH ANTIMICROBIAL ACTIVITY

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Abstract: *Aloe vera,* the popular traditional medicinal herb has tremendous history of extensive use in the treatment of hair loss, wounds, haemorrhoids and genital ulcers. Bacterial endophytes exist as symbionts in *Aloe vera* and are potential source of novel bioactive compounds. The aim of the study is to assess the antibiotic potential of cultivable bacterial endophytes, isolated from *Aloe vera* leaves using PKS and NRPS genes. The potent organisms will be screened for antimicrobial activity against gram positive and gram negative pathogenic bacteria. About twenty microorganisms were isolated from surface sterilized leaves on starch casein agar and Raffinose histidine agar plates. Four isolates with PKS or NRPS genes were identified with polymerase chain reaction screening of twenty isolates. The antimicrobial activity of the potential isolates was investigated with agar well diffusion method against four test organisms – *S aureus*, *L monocytogenes, E coli* and *P aeruginosa*. Ethyl acetate extract of *B amyloliquefaciens AVS8* exhibited broad spectrum antibiotic activity against gram positive and gram negative bacteria while the chloroform extract exhibited narrow range antimicrobial activity. The presence of PKS gene in *B amyloliquefaciens AVS8* and NRPS genes in *B licheniformis AVS10* and *B safensis AVS5* was assessed via PCR technique and are potential microorganisms for production of diverse polyketides and non ribosomal peptides.

Keywords: Aloe vera, bacterial endophytes, Non ribosomal peptide synthase, Polyketide synthase.

Introduction: There is an ever growing need for novel drugs that are useful in the treatment of cancers, microbial and fungal infections, viral and protozoan infections. Historically, natural products have played a promising role and formed the basis for most of new drugs. *Aloe vera* has been a popular choice in traditional medicine and extensively used by the Assyrians, Egyptians, Mediterranean civilizations and in Biblical times (Grindlay and Reynolds 1986). The Greek Herbal of Dioscorides describes the *Aloe vera* plant in detail and it's utilization in treatment of hair loss, wounds, hemorrhoids and genital ulcers. To date, *Aloe* is an important traditional medicine in many countries, including China, India, the West Indies, South Africa, and Japan (Grindlay and Reynolds 1986).

Compounds generated from plant preparations are principally the yields of plant metabolism. However, endophytes existing in symbiosis with plants can also produce bioactive compounds as exemplified by Taxol (Stierle *et al*, 1993; Jia-yao Li *et al*, 1996) and camptothecin (Kusari *et al* 2009; Puri *et al*, 2005) which are anticancer compounds synthesized by both endophytes and plants. Endophytes are the microorganisms that colonize the internal tissues of plants and produce secondary metabolites which confer major ecological benefits to their host plants including plant growth promotion, enhanced resistance to various predators plus pathogens (Arnold *et al.*, 2003; Zhang *et al.*, 2011) and increased drought resistance. Endophytes can produce metabolites that are similar to or the same as that produced by the host (Stierle et al, 1993). Polyketides and non ribosomal peptides, produced by microorganisms belong to diverse families of natural products and display wide range of biological activities (Staunton, J and Weissman, KJ 2001; Hopwood, DA 1997; Felnagle, E.A et al, 2008; Konz D and Marahiel MA, 1999).

Polyketides are produced by many fungi, bacteria, plants and marine organisms. Complex oligopeptides are formed by NRPSs through the linear condensation of proteinogenic and non-proteinogenic amino acids. NRPSs are involved in the biosynthesis of compounds like penicillin and cephalosporin (antibiotics), cyclosporine A (anti-inflammatory and immunosuppressive activities and fusaricidin (antibiotic). Screening for microbes with the ability to synthesize bioactive metabolites generally involves the detection of polyketide and non-ribosomal peptide synthesis pathways. There is increasing evidence that many metabolites, in particular polyketides and nonribosomal peptides, are produced by the bacterial endophytes of medicinal plants.

The aim of this study was to assess the potential of cultivable bacterial endophytes of *Aloe vera* for producing antibiotics through the detection of PKS and NRPS genes. Additionally, it also includes the identification of the bacterial isolates using 16S rRNA. It also involves the determination of inhibitory interaction between the isolates and pathogenic

organism using well diffusion assays. The use of the genetic screen increases the likelihood of isolating endophytes which biosynthesize novel pharmaceuticals independently of the host plant.

Materials And Methods:

Sample Collection, Surface sterilzation and isolation of bacterial Endophytes: Healthy leaves of *Aloe vera* plant were cut and stored in sterile plastic bags. The plant parts were carefully washed in running water to remove external soil and debris. In a method adapted from Fisher *et al* (1992), the plant material was surface sterilized by immersion in 70% ethanol for 5 min, 10% Sodium hypochlorite for 15 min, followed by washing with sterile distilled water. The material was once again subjected to 70% ethanol for 5 min and two washes with sterile distilled water. To ensure that surface sterilization was successful, sterile distilled water employed in final wash was inoculated into nutrient broth followed by incubation at 30 ^{0}C for three days and checking for any growth of plant surface-associated contaminating microorganisms. Surface sterilized plant material was cut into small pieces and subjected to homogenization under sterile environment. Homogenized serially diluted plant sample was then spread plated onto starch casein agar (Composition : Starch – 10 gL^{-1}, Cassamino acids – 0.3 gL^{-1}, $CaCO_3$ – 0.02 gL^{-1}, $FeSO_4.7H_2O$ – 0.01 gL^{-1}, KNO_3 – 2.0 gL^{-1}, $MgSO_4.7H_2O$ – 0.05 gL^{-1}, NaCl – 2.0 gL^{-1} Agar – 18 gL^{-1}) and Raffinose Histidine agar (Composition : Raffinose – 10 gL^{-1} , Histidine – 1.0 gL^{-1}, NaCl – 2.0 gL^{-1}, $FeSO_4.7H_2O$ - 0.01 gL^{-1}, $MgSO_4.7H_2O$ – 0.5 gL^{-1}, Agar – 18 gL^{-1}) followed by incubation at 30 ^{0}C for three days. After three days, distinct colonies were identified and purified on the same medium for 5 to 6 generations and stored in glycerol stocks at – 80 ^{0}C.

DNA isolation and PCR amplification of 16S rRNA genes and PKS and NRPS domain sequences: Genomic DNA was prepared from isolated bacterial strains using a modified protocol of Edward *et al*. 3-5 colonies were picked from an agar plate using a sterile toothpick and suspended in 100 µl sterile TE Buffer. Cell suspension was placed in a water bath at 97 ^{0}C for 10 minutes followed by the addition of 0.5 g glass beads (0.17 ––0.18 mm) and vortexing for 1 min. Cell lysate was centrifuged at 15000 x g for 10 min, supernatant that contains the DNA was collected and 1 µl aliquot was used for PCR. The primers and PCR conditions for the amplification of bacterial 16S rRNA, PKS and NRPS were adopted from Weisburg *et al*., 1991, Neilan, 2001 and Neilan *et al.*, 1999 respectively.

Sequence Analysis: Amplified 16S rRNA sequences of bacterial isolates were checked for similarities by BLAST searches in the NCBI databases (http://www.ncbi.nlm.nih.gov/BLAST/) and EzTaxon server 2 to seek matches with previously cultivated bacteria (Chun *et al.*, 2007). An unrooted neighbor-joining phylogenetic tree was constructed based on 16S rRNA sequences using MEGA software version 5.0.with the Poisson correction model, which also completed deletion handling of gaps and a bootstrap consisting of 1 000 replications (Zhao *et al.*, 2008). With the BlastX program in the NCBI database, the obtained PKS-KS domain (~700 bp) and NRPS-A domain (~1 000 bp) sequences were analyzed by searching for amino acid sequences similar to known PKS and NRPS sequences in Gen Bank.

Production and Crude extract Preparation: Selected bacterial endophytes were grown in 500 ml ehrlenmeyer flasks with production volume of 180 ml and incubated for four days in reciprocal shaking incubator maintained at 200 RPM and 30 ^{0}C. The production medium composition is as follows: pepsin – 5 gL^{-1}, beef extract – 1.5 gL^{-1}, Yeast extract – 1.5 gL^{-1}, starch - 10 gL^{-1}, Cassamino acids – 0.3 gL^{-1}, $CaCO_3$ – 0.02 gL^{-1}, $FeSO_4.7H_2O$ – 0.01 gL^{-1}, KNO_3 – 2.0 gL^{-1}, $MgSO_4.7H_2O$ – 0.05 gL^{-1}, NaCl – 2.0 gL^{-1}. After four days, the culture broth was centrifuged at 10000 RPM for 10 min, supernatant collected and extracted thrice with equal volumes of ethyl acetate and chloroform to give ethyl acetate and chloroform extracts respectively. Crude extracts of ethyle acetate and chloroform extracts were produced by concentration in a rota evaporator and were stored at 4 ^{0}C till use.

Antimicrobial Assays: Crude extracts were tested for antimicrobial activity using well diffusion method Perez et al., 1990) and area of zone was calculated. The tested pathogenic organisms include Staphylococcus aureus, Enterococcus faecalis, Enterobacter cloacae, Pseudomonas aeruginosa.. A broad spectrum antibiotic – Gentamicin at a concentration of 50 µg was used as a positive control.

Results And Discussion:

***Aloe vera* bacterial endophytes:** Almost every plant on the earth hosts untold number of endophytic bacteria and such bacteria from medicinally important plants are of great interest especially in understanding their potential medicinal properties and to explore their potential applications (Mehanni and Safwat 2010; Qin S et al 2011). In this study, a total of twenty bacterial endophytes exhibiting distinct colony characteristics like form, texture, opacity, surface, margin or elevation were isolated from starch casein agar and raffinose histidine agar plates. The twenty bacterial isolates were purified on the same medium and maintained as glycerol stocks stored at -80 ^{0}C. Likewise, bacterial endophytes have been reported from various medicinal plants; for examples, *Gynura procumbens, Piper nigrum, Strobilanthes crispa*, and *Vernonia amygdalina*.(Bhore et al 2010; Aravind *et al* 2009; Bhore and Tiong 2012).

However, this is the first study to elucidate different types of bacterial endophytes in *Aloe vera*

Screening PKS and NRPS genes in bacterial endophytes: PKS and NRPS genes are appropriate targets for detection of small molecule biosynthesis systems (Wawrik *et al*, 2005). The PCR screens were successful in identifying one 700 bp PKS gene from AVS8 isolate and three 1kb NRPS genes from AVS 5, AVS 8 and AVS 10 isolates. Amplification of bacterial PKS and NRPS domain sequences were confirmed via sequencing and BLASTX (translated) analysis. BLASTN (nucleotide) analysis of the PCR products did not reveal any significant matches to the nucleotide database, indicating that the sequences were novel at this level. BLASTX analysis revealed that the predicted translated DNA sequences were homologous to bacterial PKS and bacterial NRPSs, with sequence similarity between 58% and 68%.

Identification of bacterial endophytes: Phylogenetic analysis of 16S rRNA gene sequences indicated the three strains positive for PKS and NRPS genes belong to the family *Bacillaceae*. They showed high identity (>98%) to species in genus *Bacillus* and exhibited 91%–99% identity to each other. BLAST searches in the NCBI database and EzTaxonZ server showed that the isolates AVS5, AVS8 and AVS10 were identified to be *Bacillus safensis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* respectively. Bacillus bacteria living as endosymboints in various medicinal plants, could play important role as sources of polypeptide antibiotics (Lebbadi *et al*., 1994; Hathout *et al*., 2000).

Antimicrobial Activity: Screening for new antibiotics from natural sources is becoming increasingly important for the pharmaceutical industry as pathogenic bacteria are increasingly becoming resistant to commonly used therapeutic agents. The results in Table 1 and Fig 2 indicates that *Bacillus amyloliquefaciens AVS8* produced good inhibitory effect against gram positive and gram negative bacteria as noticed against *S aureus*, *L monocytogenes*, *E coli* and *E aerogenes*.

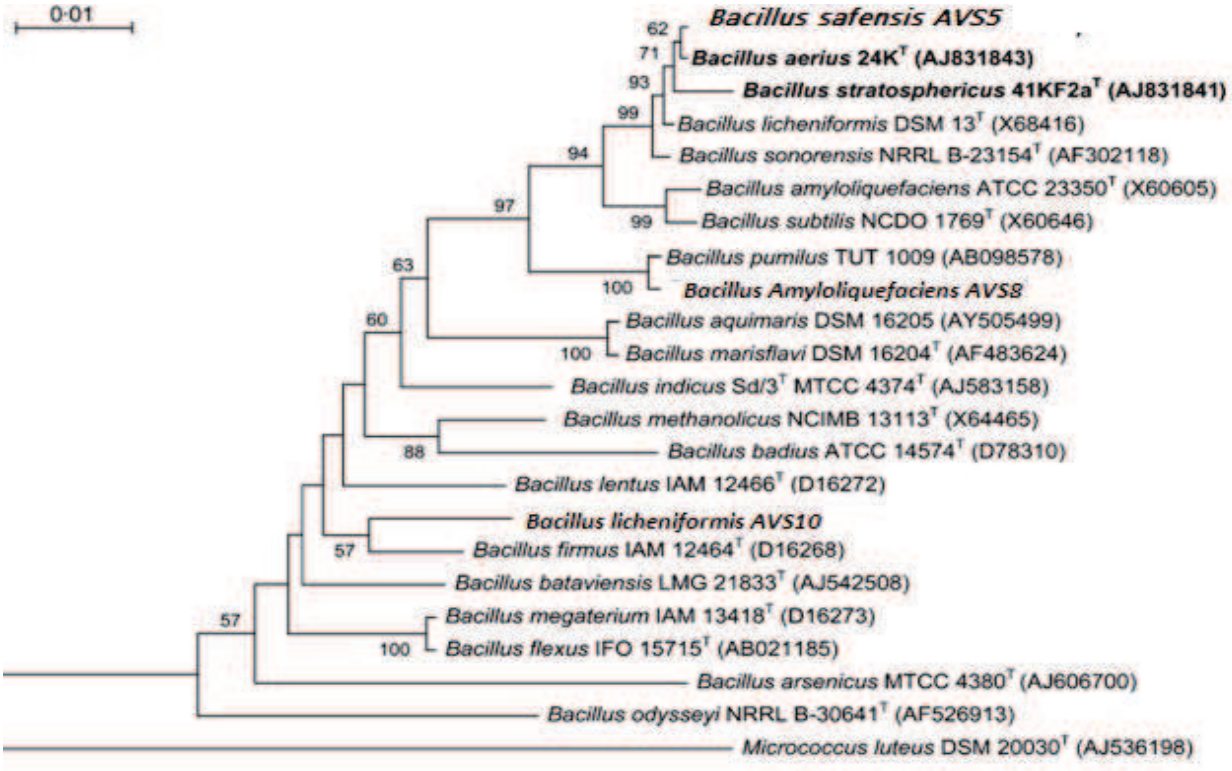

Fig 1 Phylogenetic tree showing the relationship among the identified bacterial endophytes.

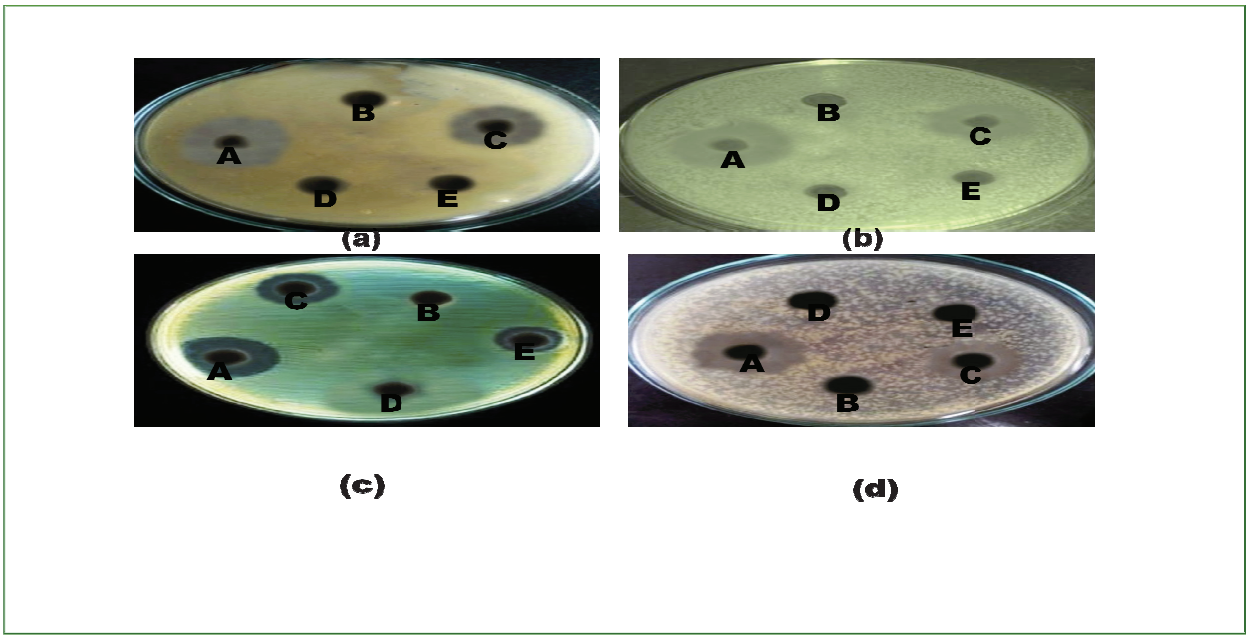

Fig 2: Antimicrobial activity of ethyl acetate and chloroform extracts of *B amyloliquefaciens AVS8.* (a) *Staphylococcus aureus* (b) *Listeria monocytogenes* (c) *E coli* (d) *E aerogenes*; A – Positive control, B – Ethyl acetate, C – Ethyl acetate extract, D – Chloroform, E – Chloroform extract.

Table 1: Zone of inhibition measurements made against the pathogenic organisms.

Pathogenic Organism	Zone of Inhibition (mm)		Positive Control
	Ethyl acetate extract	Chloroform extract	
Staphylococcus aureus	13.32±0.74	absent	29.37±0.68
Listeria monocytogenes	11.54±0.58	absent	26.48±0.72
Escherichia coli	9.34±0.52	7.32±0.66	31.52±0.56
Enterobacter aerogenes	8.95±0.71	absent	28.42±0.61

Bacillus species, the largest antibiotic producers, represent a rich source of structurally diverse secondary metabolites such as lipopeptides, macrolactones, polyketides etc as evident by potential reviews (Hamdache *et al.*, 2011; Stein, 2005; Kim *et al*, 2003). This investigation has revealed the potentials of *Bacillus* species especially in antibiotic production. It is therefore important to isolate, purify and characterize the chemical nature of the antibiotic.

Conclusion: The bacterial endophyte isolated from *Aloe vera, Bacillus amylofaciens* AVS8 exhibits antibacterial activity. The crude extract of this strain was found to be active against both gram positive and gram negative pathogenic bacteria. *Bacillus amylofaciens* AVS8 has the potential for the production of antibiotics in future.

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