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Review

Review on bioactive potential of marine microbes

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There is a need for new drugs especially novel antibiotics, anticancer compound, antibiotic against multi drug-resistant bacteria. The discovery and development of new drugs from marine microbes played a significant role over the last few decades. The world oceans comprise a rich diversity of microbial life with current estimates reaching over a million different species. Marine microbes are important from both ecological and biotechnological point of view. Marine microbial bioactive compounds have attracted increasing attention from microbiologists, taxonomists, ecologists, agronomists, chemists and evolutionary biologists.

Key words: Bioactive molecules, bacteria, fungi.

INTRODUCTION

Marine microbes having immense genetic and bio-chemical diversity have become a rich source of novel effective drugs. It is surprising to find that many bioactive compounds, reported from marine invertebrates are produced by their microbial symbionts. Competition among microbes for space and nutrients in the marine environment is a driving force behind the production of such precious antibiotics and other useful pharma-ceuticals. Interestingly, microorganisms associated with marine invertebrates are proved as valuable candidates for drug discovery program.

Like bacteria, marine fungi are also reported to be potential source of bioactive substances. For example, sorbicilactone-A, a novel type alkaloid was reported from sponge (*Ircinia fasciculata)* associated fungus, *Penicillium chrysogenum*. This compound showed thera-peutic potential in human trials. Polyketide synthases (PKSs) are a class of enzymes that are involved in the biosynthesis of secondary metabolites like erythromycin (Lamela et al., 1989; Parkes et al., 1994; Hentschel et al., 2003).

Since the discovery of penicillin in 1929, a number of natural products are reported to have biological activities that are in use today as antibiotics, antitumour agents and agrochemicals (Davidson et al., 2001). The development of the fungal metabolite, Mevinolin for the treatment of high serum cholesterol and the bacterial metabolite FK-506 as an immunosuppressant illustrate the vast natural diversity of microbial natural products (Faulkner, 1992). In spite of such successes in drug discovery from terrestrial microorganisms, marine microorganisms have received only very little attention on account of the non-culturability of the majority of marine bacteria (Gaino and Sara, 1994).

Marine toxins such as tetrodotoxin and saxitoxin are potent and specific sodium channel blockers and pharmacological studies on these toxins have played a major role in developing the concept of sodium channels in general and membrane channels in particular (Gustafson et al*.,* 1989). There is an increasing evidence that several anti-cancer compounds isolated from sponges or other marine invertebrates are actually produced by bacteria associated with these invertebrates. Bryostatins were initially isolated from a bryozoan, *Bugula neritina.* Putative type 1 polyketide synthase genes had been found in bacteria from colonies of *B. neritina* producing bryostatin and these genes were absent in bacteria associated with colonies of *B. neritina*

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that did not produce bryostatin (Holst et al., 1994). Dolastatin isolated from the Indian Ocean seahare *Dolabella auricularia* has also been isolated from marine cyanobacteria (Hugenholtz and Pace, 1996) like *Lyngbya majuscule* (Ireland, 1993).

MARINE BACTERIA AND ACTINOMYCETES

About 20% of bacteria from marine sponge and coral in different coastal areas of the China Sea showed positive antimicrobial activity (Zheng et al., 2005). In the study of Li et al. (2007), some bacteria were isolated from the sponges *Stelletta tenuis*, *Halichondria rugosa* and *Dysidea avara* which showed pronounced broad-spectrum of antimicrobial activities and enzymatic potentials. Brammavidhya and Usharani (2013) extracted and optimized the bioactive compound and it act against the human pathogen which was isolated from *Hyattella cribriformis* associated *Bacillus cereus* SBS02.

The major antimicrobial metabolite isolated from *Pseudoalteromonas piscicida* NJ6-3-1 with wide antimicrobial spectrum was identified as norharman (a beta-carboline alkaloid) (Zheng et al., 2005). Cyclo-L-Phenylalanyl-L-Proline (Cyclo-L-Ph- L-Pro) was isolated from *Alcaligenes faecalis* A72 associated with the sponge *S. tenuis* which showed moderate inhibitory activity against *Staphylococcus aureus* (Li et al., 2008). Bioassay-guided fractionation of the $CHCl_3$ extract of the fermentation broth of a sponge *Mycale plumose*-derived actinomycete *Saccharopolyspora* spp. nov., led to the isolation of two known prodigiosin analogs and undecyl prodigiosin. These compounds exhibited significant cytotoxic activities against cancer cell lines: P388, HL60, A-549, BEL-7402 and SPCA4 (Liu et al., 2005). Revathy et al. (2013) studied the antioxidant and enzyme inhibitory potential of *Streptomyces* sp. VITMSS05 strain, isolated from Marakkanam, southern coast of India.

Bacillamides have been proven to inhibit the growth of red-tide algae such as *Cochlodinium polykrikoides* (Jeong et al., 2003). A novel thiazole alkaloid, neobacillamide A together with a known related one, bacillamide C were isolated from *Bacillus vallismortis* C89 associated with the South China Sea sponge, *Dysidea avara* (Yu et al., 2009). Studies made by the scientists at the Scripps Institute of Oceanography showed that marine bacteria are capable of producing unusual bioactive compounds that are not observed in terrestrial sources (Fenical, 1993; Fenical and Jensen, 1993). Pandey et al. (2013) studied the diversity of beta-glucosidase inhibitors producing marine bacteria. They observed that the marine sponge *Aka coralliphaga* associated bacteria had produced beta-glucosidase inhibitors as compared to other associated microbes. Mithun and Rama (2012) isolated and identified *Micrococcus luteus* sp. from marine soil samples collected from Bay of Bengal coast of Machilipatnam, Krishna district, Andhra, India. This strain showed promising results against cancer cell line namely HCT 15 and MES-SA.

Thermo-stable proteases, lipases, esterases and starch and xylan degrading enzymes have been actively sought and in many cases are found in bacterial and archaeal hyper thermophilic marine microorganisms (Bertoldo and Antranikian, 2002). An unusual Gram-positive bacterium from deep-sea sediment produced a series of natural products, for example macrolactin A-F of an unprecedented C_{24} linear acetogen origin (Gustafson, 1989). The major metabolite, macrolactin A inhibited B16-F10 murine melanoma cells in *in vitro* assays, showing significant inhibition of mammalian Herpes simplex virus (type I and II) and protecting T lymphocytes against human immunodeficiency virus (HIV) replication (Carte, 1996).

On the other hand, a microbial metabolite obtained from *Alteromonas* spp. isolated from the tissues of sponge had anti HIV activity due to the production of reverse transcriptase inhibitor. Some *Vibrio* species have been found to produce a variety of extra cellular proteases. *Vibrio alginolyticus* produced six proteases including an unusual detergent-resistant, alkaline serine exoprotease, this marine bacterium also produced collagenase, an enzyme with a variety of industrial and commercial applications, including the dispersion of cells in tissue culture studies (Graham et al., 1980; Osama and Koga, 1995). Bioactive compound, Cis-sativenediol extracted from *Aeromonas* sp and *Rhodopseudomonas* sp. which was collected from coastal water of Thoothukudi, Thiruchendur and Kanyakumarai Tamil Nadu, India act against *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Staphylococcus epidermidis* and *S. aureus* (Ashadevi et al., 2011).

Pyridinium, a compound with antimicrobial activity was isolated from marine actinomycete, *Amycolatopsis alba* var. nov. DVR D4 (Dasari et al., 2012). Actinomycetes from marine sediment have anticancer activity against breast cancer cell lines MCF-7 and MDA-MB-231 (Ravikumar et al., 2012). The cytotoxicity and antioxidant activity of 5-(2,4-dimethylbenzyl)pyrrolidin-2-one (DMBPO) extracted from marine *Streptomyces* VITSVK5 sp. from sediment samples collected from the Marakkanam coast, Bay of Bengal, India was studied (Saurav and Kannabiran, 2012). Anthracyclinones namely 4,6,11-trihydroxy-9-propyltetracene-5,12-dione and 10β-carbomethoxy-7,8,9,10-tetrahydro-4,6,7α,9α,11-pentahydroxy-9-propyltetracene-5,12-dione have cytotoxic activity against the HCT-8 human colon adenocarcinoma cell line, which were isolated from *Micromonospora* sp. associated with the tunicate *Eudistoma vannamei* (Sousa et al., 2012). Sivasankar et al. (2013) isolated and optimized the l-asparginase from marine *Streptomyces* sp. which showed the antibacterial activity.

MARINE FUNGI

A compound, (*S*)-2,4-dihydroxy-1-butyl(4-hydroxy) benzoate and another compound, fructigenines A exhibiting cytotoxic activity against FT210 cells, were isolated from fungus *Penicillium auratiogriseum* derived from sponge *Mycale plumose* (Xin et al*.,* 2005). Xie et al*.* (1996) demonstrated that two antifungal trichothecenes, including roridin A and roridin D produced by the fungus *Myrothecium* spp. isolated from the marine sponge *Axinella spp*. in the South China Sea, could be potential inhibitors against the plant pathogen *Sclerotinia sclerotiorum* The sponge *Acanthella cavernosa*-associated fungus, *Letendraea helminthicola*, produced two antifouling compounds such as 3-methyl-*N*-(2-phenylethyl) butanamide and carbo methoxycarbonyl -D-Pro-D-Phe benzyl ester (Yang et al., 2007) .

Three quinazoline alkaloids, aurantiomides A, B, and C were isolated from *Penicillium aurantiogriseum* SP0-19 associated with sponge *Mycale plumose* among which aurantiomides B and C showed moderate cytotoxicities against HL-60, P388 and BEL-7402 and P388 cell lines, respectively (Xin et al., 2007).

Beside sponges, some pharmaceutical metabolites have been isolated from marine microbial symbionts associated with sea squirts, marine bryozoans and molluscs. 5α,8α-epidioxy-23-methyl-(22*E*,24*R*)-ergosta-6,22-dien-3β-ol with cytotoxic activity was isolated from the fungus *Penicillium stoloniferum* QY2-10, associated with a sea squirt (Xin et al*.,* 2007). Six new ergo sterols obtained from a marine *Bugula* spp. derived fungus *Rhizopus* spp. showed activities against P388 and HL-60, A549 and BEL-7402.

Seven new prenylated indole diketopiperazine alkaloids, including spirotryprostatins derivatives of fumitremorgin B, and 13-oxoverruculogen, have been isolated from the holothurian-derived fungus *Aspergillus fumigatus*. These compounds showed anticancer activity to four cancer cell lines, MOLT-4, A549, HL-60 and BEL-7420 (Wang et al., 2008). Lasiodiplodin, which could inhibit the *in vitro* growth of *S. aureus*, *Bacillus subtilis* and *Fusarium oxysporum,* were isolated from the mycelium extracts of an endophytic fungus obtained from the brown algae species collected from Zhanjiang sea area by Yang et al*.* (2006).

When compared with the small molecular metabolites of marine microbial symbionts, the investigation of pharmaceutical big molecule metabolites is rare. The gene cloning, purification, properties, kinetics and antifungal activity of chitinase from marine *Streptomyces* spp. DA11 associated with the South China Sea sponge *Craniella australiensis* were investigated by Han et al. (2009).

Association of fungi with marine animals ranges from saprotrophic, symbiotic to parasitic. Saprotrophic fungi have been isolated from the surface, guts and fluids of holothurians or the sea cucumbers (Pivkin, 2000).

Several fungi isolated from invertebrates have been found to produce interesting secondary metabolites. A novel group of platelet activating factor (PAF) antagonists, phomactins A, B, B1 and B2 were isolated from culture broth of *Phoma* isolated from the shell of crab *Chinoecetes opilio* collected from the coast of Fukuii, Japan (Sugano et al., 1991). Two lipophilic tri peptides from *Penicillium fellutanum* live inside the gastrointestine of a marine fish and three quinazoline derivatives from *Aspergillus fumigatus* from gastrointestinal tract of the fish, *Pseudolabrus japonicas* from the coast of Japan (Kobayashi and Ishibashi, 1993). An endolithic fungus *Ostracoblabe implexa* living inside the shells of rock oyster *Crassostrea cucullata* from the coast of Goa was reported (Raghukumar and Lande, 1988). Fungi belonging to the class of Trichomycetes are found in the guts of marine arthropods, isopods, decapods and amphipods in a symbiotic association (Misra and Lichtwardt, 2000). Endolithic fungi in coral skeleton are common (Alsumard et al., 1995; Ravindran et al., 2001; Golubic et al., 2005).

A basidiomycetous yeast *Cryptococcus* spp. associated with *Pocillopora damicornis* coral skeleton was shown to produce a transient cryoprotective effect, selectively enhancing the survival of skeletogenic cell types (Coulon et al., 2004) - which gave some detailed characteristic of this cryoprotective effect, some values would be very interesting. *Colecobasidium* species was isolated from necrotic patches in several massive corals in the Andamans (Raghukumar and Raghukumar, 1991).

Biological activities of microbes are mainly focused in the areas of antibiotic and anticancer properties, but other selective activities include cell cycle inhibition, antagonism of platelet activating factor, antiviral activity, neuritogenic activity, phosphatase inhibition and kinase inhibition and radical scavenging activities are less attended (Bugni and Ireland, 2004). Among the eukaryotic microscopic fungi, the inhibitory compounds capability of imperfect fungi, the ascomycetes and several other filamentous and endophytic fungal species are the most significant (Shearer and Maivan, 1988). Several compounds that inhibit the growth of a large spectrum of saprophytic and phytopathogenic fungi were isolated from basidiomycetes (Anke, 1989, 1995).

A marine-derived *Penicillium chrysogenum* isolated from the sponge, *Ircinia fasciculate* produced a sorbicillin derived alkaloid sorbicillacton A (Bringmann et al., 2003) which showed selective cyctostatic activity against lympho blasts and was able to protect human T cells against HIV-1. Preclinical evaluation of this compound has recently been initiated. Many of these fungi have novel carbon skeletons thus providing further evidence of the potential of marine-derived fungi (Proksch et al., 2003).

The first crystalline fungal product obtained from *Penicillium glaucoma* was chemically myco phenolic acid and it was discovered in 1896 (Berdy, 2005). Microbial

secondary metabolites regulate growth processes, replications, and/or exhibited some kind of regulatory, inhibitory or stimulatory responses in prokaryotic and eukaryotic cells even at a minimal concentration. Marine-derived isolate of *Aspergillus versicolor* (MST-MF495) yielded many known fungal metabolites like sterigmatocystin, violaceol I, violaceol II, diorcinol, (-) cyclopenol and viridicatol, along with a new alkaloid, cottoquinazoline A , and two new cyclopentapeptides, cotteslosins A and B (Fremlin et al*.,* 2009). Moreover, the great practical and historical importance of beta-lactams (penicillins, cephalosporins), the cyclosporin, and various statins (mevinolin, compactin, lovastatin, pravastatin, atrovastatin) which are all fungus derived compounds are well known (Donadio et al., 2002).

Several compounds from marine fungi have been screened for antifungal activities, and a number of compounds have been characterized with regard to their inhibitory activities and chemical structures. There has been a sharp increase in fungal infections among patients suffering from HIV, receiving cancer and immune-therapy (Anaissie, 1992; Li et al*.,* 1998; Barrett, 2002). Recent research showed that compounds like Hypoxysordarin, a new sordarin derivative, isolated from the facultative marine fungus *Hypoxylon croceum* are promising.

Terrestrial fungi produce a variety of chemically diverse natural compounds with interesting biological activities. Until recently, only modest attention has been given to metabolites from marine (marine derived) fungi. To date, a number of new compounds from marine fungi have been described and reviewed (Biabani and Laatsch, 1998; Faulkner, 2000, 1992; Jensen and Fenical, 2002; Proksch et al., 2003; Bugni and Ireland, 2004). These documented discoveries clearly showed that marine fungi are of high profile source of structurally unique and biologically active natural compounds as compared to terrestrial fungi. Fungi associated with sponges are the single most potential source of new marine fungal compounds and display diverse biological activities (Jensen and Fenical, 2000; Bugni and Ireland, 2004). These fungi account for the largest number of total described marine fungal compounds and produce highest number of novel metabolites (Bugni and Ireland, 2004).

Encouraged by the idea of “Drugs from the Sea”, the chemists have identified many bioactive compounds with novel structures from marine bio-resources in recent years (Faulkner, 2000; Marris, 2006). Among them, marine derived fungi have contributed an important proportion. Many marine fungal strains were isolated, screened and reported to produce novel antimicrobial compounds like alkaloids, macrolides, terpenoids, peptide derivatives and other types (Bugni and Ireland, 2004; Saleem et al., 2007). These marine fungal derived compounds have provided us with new choices to fight infectious diseases. For example, the marine fungus *Pestalotia* spp. isolated from the surface of the brown alga *Rosenvingea* spp. was able to produce a new chlorinated benzophenone compound pestalone, which showed potent antibiotic activity against multi drug resistance *Staphylococous aureus* (MRSA), revealing its potential as a new antibiotic. Current studies indicate that complex interactions exist between the host and their epi/endophytic fungi, e.g. the host provides organic nutrition and epi/endophytes act as chemical guards (Tan and Zou, 2001; Bugni and Ireland, 2004). As compared to free-living marine fungi, the epi/endophytic marine fungi have drawn more interest of natural product chemists in search for novel antimicrobial or other active compounds. According to Bugni and Ireland (2004) among the new compounds reported from marine derived fungi till 2002, most of them are produced by epi/endophytes isolated from plants, invertebrates and woody substrates.

CONCLUSION

The world oceans comprise a rich diversity of microbial life with current estimates reaching over a million different species. The vast metabolic diversity of marine microorganisms is underpinned by novel enzymatic functions. While marine microorganisms have already proven to be a rich source of biologically active compounds (bioactives), recent large-scale surveys indicated the existence of thousands of other yet undescribed protein families. Chemically mediated interactions and communications between the microorganisms and their eukaryotic hosts are also likely to have a significant impact on the composition and function of surface consortia. These factors shape the composition and properties of the surface community. The ocean represents a rich resource of even more novel compounds with great potential as pharmaceutical, nutritional supplements, cosmetics, agrichemicals and enzymes, where each of these marine bioproducts have a strong potential market value.

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Full Length Research Paper

Siderophore mediated antagonism of fluorescent Pseudomonads against soil borne plant pathogenic fungi in West Bengal, India

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The rhizobacterial isolates from different agro ecological region of West Bengal, used in this study, were identified as fluorescent pseudomonads based on biochemical and molecular characterization. Six effective antagonist isolates were selected for further studies of secondary metabolite (siderophore) production with respect to bacterial growth at different time intervals (6 to 96 h), the nature of siderophore production (hydroxamate and/or catecholate type), effect of iron supplementation at its different concentration on siderophore production and antagonistic potentiality. Observations of higher level of growth and siderophore production, for most of the isolates, were recorded after 72 h of incubation. The maximum zone of inhibition and siderophore production was recorded at 0.5 µM and the least at 10 µM iron concentration. With the gradual increase in iron supplementation in king's B medium, the siderophore production as well as *in vitro* antagonistic potentiality decreased in terms of enhancement of diameter of fungal mycelium and reduction of percent inhibition of pathogen by fluorescent pseudomonads in dual culture assay method.

Key words: Fluorescent pseudomonads, siderophore, ferric ion, *Sclerotium rolfsii, Rhizoctonia solani*.

INTRODUCTION

Many genera of plant root-inhabiting bacteria have potential as biological control agents (Weller, 1988). The genus *Pseudomonas* belongs to the γ-subclass of the Proteobacteria and includes mostly fluorescent Pseudomonads as well as a few non-fluorescent species. Particularly fluorescent *Pseudomonas* spp. has received much attention because of their suggested involvement in natural disease suppressiveness of certain soils (Weller et al., 2002). Iron-regulated metabolites are mainly referred to as siderophores. The siderophores are high-affinity iron chelators in bacteria, enabling uptake of Fe (Braun, 2001). Iron is essential for the growth of almost all organisms but, in many environments, the amount of free iron is below 10^{-7} M, which is the required concentration by most bacteria for growth (Ratledge and Dover, 2000). Bacteria have developed several strategies for the acquisition, solubilization and transportation of iron (Guérinot, 1994). The most efficient mechanism of iron acquisition is the secretion of high affinity iron-chelating compounds, so-called siderophores. The siderophores chelate iron in the extracellular environment and the resulting ferric siderophore complex is recognized by

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Table 1. Origins and different crop rhizospheres of the isolates.

Fluorescent pseudomonad isolates	Location	Region	Crop rhizosphere
MFP9	Coochbehar	Terai	Maize
CHFP23	Nadia	Gangetic alluvial	Chilli
TFP16	West Midnapore	Lateritic	Tomato
FPK7	Bagdaha, North 24Pgs	Gangetic alluvial	Pointed gourd
FPK8	Bagdaha, North 24Pgs	Gangetic alluvial	Pointed gourd
FPK10	Habra, North 24 Pgs	Gangetic alluvial	Cucumber

siderophore-specific membrane receptors, enabling uptake of Fe by the bacterial cells (Braun, 2001). Many bacteria produce more than one type of siderophore, as shown for *Enterobacter cloacae*, *Mycobacterium smegmatis* and *Pseudomonas* spp. (Adilakshmi et al., 2000; Mercado-Blanco et al., 2001). There are several reports where the effective role of siderophore on antagonistic potentiality under *in vitro* and *in vivo* cinditions, has clearly been discussed (Pal and Gokarn, 2010; Adhikari et al., 2013; Subashri et al., 2013). Although considerable structural variation exists among the several dozen siderophores chemically characterized at the present time, most can be classified as hydroxamates or catechols (Neiland, 1981). The present study on siderophore producing fluorescent pseudomonads was concentrated on the screening for their siderophoregenesis, siderotyping and siderophore mediated antibiosis under *in-vitro* conditions against pathogenic fungi with different concentration of iron supplement.

MATERIALS AND METHODS

Isolation of bacteria

Soil samples were collected from different crop rhizospheres from different agro-ecological region of West Bengal (Table 1). Roots were shaken vigorously to remove loosely adhering rhizospheric soil. To isolate rhizospheric bacteria, 10 g of rhizospheric soil were shaken in 90 ml sterile phosphate buffer saline (PBS) and were four fold diluted and plated on King's B (KB) medium. A total of 100 bacterial isolates were isolated and fluorescent colonies, screened through UV light (k = 365 nm), with different morphological characteristics were subcultured by repeated streaking on KB medium. On the basis of siderophore production property five highly and one moderately siderophore producing isolates were selected for further studies.

Characterization of siderophore producing bacteria

Bacterial identification was done by biochemical analysis on the basis of the tests of oxidase, arginine dihydrolase, denitrificacation, gelatine hydrolysis, levan production, and acid production from trehalose, utilization of tryptophane and L-tartrate coupled with amplification of the *Pseudomonas* genus specific 618 bp fragment of 16S rDNA genes by PCR using the primers PA-GS-F and PA-GS-R corresponding to the 5' (5'-GACGGGTGAGTAATGCCTA-3') end and 3' end (5'-CACTGGTGTTCCTTCCTATA-3') of the 16S rDNA gene (Spilker et al., 2004). Amplification was confirmed by analyzing 5uL of each PCR reaction mixture on a 1% agarose gel (Sambrook et al., 1989).

Screening of Siderophore producing bacteria

Qualitative assay

Siderophore production by all the isolates was tested qualitatively by Chrome Azural S (CAS) plate assay (Schwyn and Neilands, 1987). The isolates were spotted over cetrimide agar and incubated for 48 h at 28°C. After incubation, a thin layer of CAS reagent in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24 h at 28°C, formation of yellow orange color zone around the colonies in plate assay indicated the siderophore production.

Quantitative assay

All the isolates were grown at 28±2°C on a rotary shaker in King's B broth for three days and centrifuged at 10,000 rpm for 10 min and the supernatant was collected. The pH of the supernatant was adjusted to 2.0 with 1 N HCl and equal quantity of ethyl acetate was added in a separating funnel, mixed well and ethyl acetate fraction was collected. 5 mL of ethyl acetate fraction was mixed with 5 mL of Hathway's reagent (The reagent was prepared by adding 1 mL of 0.1 M ferric chloride in 0.1 N HCl to 100 mL of distilled water, and to this 1 mL of 0.1 M potassium ferricyanide was added). The absorbance for dihydroxy phenols was read at 700 nm in a Spectrophotometer (Reeves et al*.,* 1983). A standard curve was prepared using different concentration of dihydroxy benzoic acid at 700 nm. The quantity of siderophore synthesized was expressed as µ mol benzoic acid mL^{-1} of culture filtrate.

Siderophore typing

Hydroxamate type of siderophore

Csaky's test: Hydroxamate type of siderophore was determined by hydrolyzing 1 ml supernatant of overnight grown culture with 1 ml of 6 N H_2SO_4 in boiling water bath for 6 h or 130°C for 30 min. Further, this hydrolyzed sample was buffered by adding 3 ml of sodium acetate solution. To this, 0.5 ml iodine was added and allowed to react for 3-5 min. After completion of reaction, the excess iodine was destroyed with 1 ml of sodium arsenate solution. Finally 1 ml of α - Naphthylamine solution was added and allowed to develop the colour. Wine red colour formation indicates production of hydroxamate type of siderophore (Gillan et al., 1981).

Neilands spectrophotometric assay

The hydroxamate nature of siderophore was detected by Neilands spectrophotometric assay (Jalal and Vander Helm, 1990; (Neilands, 1981) where a peak between 420-450 nm on addition of 2% aqueous solution of $FeCl_3$ to 1 mL of supernatant indicated presence of Ferrate hydroxamate.

Catecholate type of siderophore

Arnow's Test

To 1 ml of cell-free supernatant, 1 ml of nitrite-molybdate reagent with 1 ml NaoH solution was added. Finally, 1 ml of 0.5 N HCl was added and allowed to develop colour. Yellow colour formation indicates production of catecholate type siderophore (Arnow, 1937).

Spectrophotometric assay

Catecholate nature of siderophore was detected by the method of Jalal and Vander Helm (1990) using spectrophotometric assay where a peak at 495 nm on addition of 2% aqueous solution of $FeCl_3$ to 1 mL of supernatant indicated the presence of siderophores of catecholate nature.

Temporal variation of bacterial growth and siderophore production

All the six fluorescent pseudomonads were grown in king's B medium by submerged culture method in 50 mL conical flasks and incubated at 28±2°C on a rotary shaker at 150 rpm. Samples were withdrawn in duplicate at definite time intervals from 6 h up to 96 h. One set was used for growth measurement at 600 nm and the other for estimation of siderophore at 700 nm (Reeves et al., 1983).

Influence of iron on production of siderophore

In order to determine the concentration of iron at which siderophore biosynthesis is minimized in fluorescent pseudomonads under study, all the cultures were grown in king's B media, externally supplemented with different concentrations of iron ($FeCl_3$. $6H_2O$) viz., 0.5, 1, 1.5, 2.5, 5 and 10 µM. Following the incubation at 28±2°C for 72 h at 120 rpm, siderophore contents were estimated at 700 nm. To remove traces of iron, glasswares were cleaned with 6 M HCl and with double distilled water.

In vitro antagonistic effect on mycelial growth of phytopathogen under different iron limiting conditions

An agar plug (7 mm diameter) taken from actively growing fungal culture such as *Rhizoctonia solani* and *Sclerotium rolfsii* were placed on the surface of the plate-enhancing medium supplemented with $FeCl_3$ at different concentration that is 0.5, 1, 1.5, 2.5, 5, 10 µM. Simultaneously, fluorescent pseudomonas isolates were streaked 3 cm away from the agar plug at both sides towards the edge of Petri plates (Kumar et al*.,* 2000). Plates inoculated with fungal agar plugs alone were used as control. The plates were incubated at 28±2°C until fungal mycelia completely covered the agar surface in control plate. Observations on mycelial growth of test pathogens were recorded and percent inhibition of pathogen growth were calculated:

$$\%I = 100(C-T)/C$$

Where, I = inhibition of mycelial growth, C = growth of pathogen in the control plate (cm) and T = growth of pathogen in dual cultures (cm).

Statistical analysis

All the isolates were clustered with respect to their siderophoregenesis at different ferric ion concentration, zone of inhibition, inhibition percentage, temporal growth measurement and siderophore production at different time intervals by critical difference at 5% probability level using DMRT by SPSS v.10.

RESULTS AND DISCUSSION

Characterization of siderophore producing bacteria

Biochemical characterization

Among the bacterial isolates, five highly and one moderately siderophore producing isolates were confirmed as fluorescent pseudomonads based on biochemical tests such as gram reaction, oxidase, arginine dihydrolase, denitrificacation, gelatine hydrolysis, levan production, and acid production from trehalose, utilization of tryptophane and L-tartrate. Microscopic observation showed that all the six isolates were rod shaped, motile, gram negative, and fluorescent in the presence of UV light (Rao et al*.,* 1999; Srivastava et al*.,* 1999). All the isolates exhibited positive reaction in catalase, oxidase, arginine di hydrolase and negative in TABAC hypersensitivity test (Bossis et al*.,* 2000). All the five isolates except FPK 10 were positive in gelatin liquefaction test; isolates MFP9 and FPK7 showed positive in trehalose test; MFP9 was positive in levan production and FPK7, FPK8 showed denitrification activity; whereas, the isolate TFP16 exhibited positive reaction in all the cases except TABAC hypersensitivity (Table 2).

Molecular characterization

PCR assays employing genus specific primer pair PA-GS-F/PA-GS-R produced 618 bp DNA fragments of the predicted size for all the six isolates (Figure 1). With the presence of the 618 bp amplicon the six putative siderophore producing isolates were confirmed as *Pseudomonas sp*. (Spilker et al*.,* 2004). All the isolates produced diffusible fluorescent pigment which was confirmed in the presence of UV light, which indicated that all the isolates belonged to fluorescent pseudo-monads.

Qualitative assay for siderophore production

All the six isolates were subjected to screening for side-

Table 2. Different biochemical tests performed to identify the isolates.

Fluorescent pseudomonad isolates	Fluorescence	Arginine	Oxidase	catalase	Tabac	Gelatine	Trehalose	Levan	Denitrification	L-ara	L(+)tart
MFP9	+	+	+	+	-	+	+	+	-	NA	NA
CHFP23	+	+	+	+	-	+	-	-	-	NA	NA
TFP16	+	+	+	+	-	+	+	+	+	+	+
FPK7	+	+	+	+	-	+	+	-	+	NA	NA
FPK8	+	+	+	+	-	+	-	-	+	NA	NA
FPK10	+	+	+	+	-	-	-	NA	NA	NA	NA

'+' indicates positive; '-' indicates negative; 'NA' indicates not applicable; 'L-ara' indicates L- arabinose; 'L (+) tart' indicates L-tartarate.

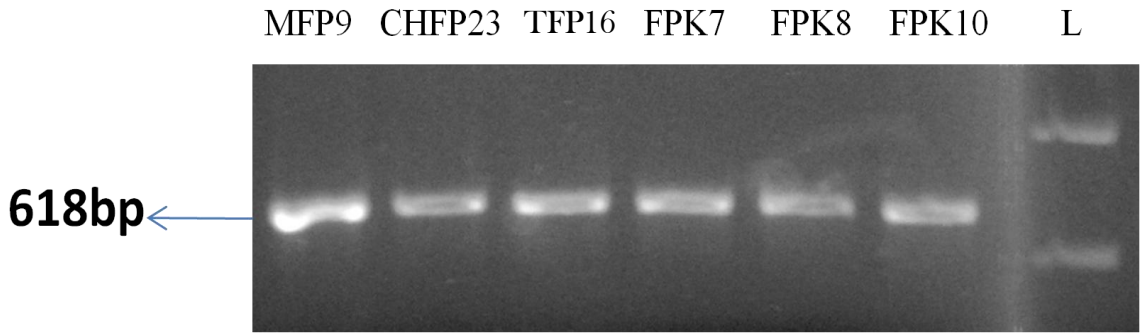

Figure 1. Agarose gel electrophoresis of PCR amplified genus specific 618 bp 16S rDNA amplicons; Lanes are named with respective isolates; L: 100 bp molecular marker.

rophore production. Isolates CHFP23, FPK8, FPK10 produced a strong reaction with the CAS reagent (Table 3). After incubating for a period of 24 h in the dark at 28°C, a change in color from blue to orange was observed in the agar plate (Figure 2). The color change from blue to orange resulted by siderophoretic removal of Fe from the dye.

Quantitative assay for siderophore production

All the six isolates were subjected to screening for siderophore production. Three isolates, viz., FPK8, FPK10, CHFP23, were found to be highly siderophore producer such as 15.20 (± 0.33), 13.9 (± 0.30) and 11.55 (± 0.21), µM/ml respectively, whereas, moderately siderophore production was obtained from the isolates TFP16 and FPK7 and the least siderophore production was observed in MFP9 (Table 4).

The siderophores have very high affinity for ferric iron and form ferric-siderophores complex making it unavailable to other organisms but the producer organism can utilize these complexes via a specific receptor in their outer cell membrane (Buyer and Leong, 1986). Fluorescent pseudomonads produce several siderophores such as pyoverdine, pyochelin (Dave and Dube, 2000) and are important to agriculture because they can reduce crop yield losses caused by bacteria and fungi in the root environment. The protective activity is related to the production and excretion of siderophores which efficiently chelate the iron in the root environment. This iron deficiency leads to an impaired growth of the deleterious micro-organisms.

Table 3. Sideropore production under *in vitro* condition by six different fluorescent pseudomonads.

Fluorescent pseudomonad isolates	Siderophore production in CAS agar plate assay
MFP9	+
CHFP23	+++
TFP16	++
FPK7	++
FPK8	+++
FPK10	+++

'+' indicates weak producer; '++' indicates medium producer and '+++' indicates good producer.

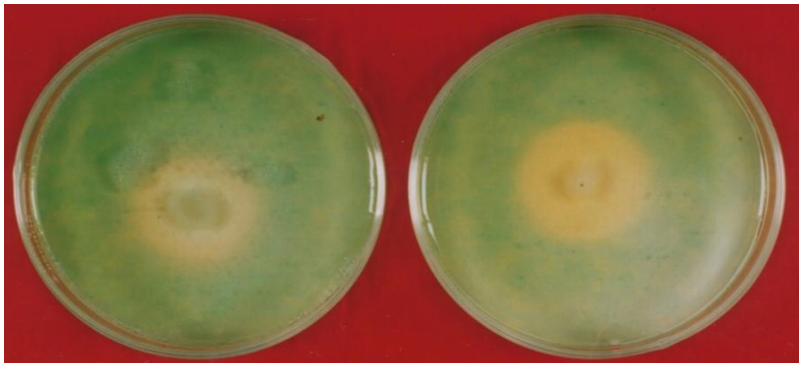

Figure 2. Siderophore production by bacterial isolates in CAS plate assay.

Table 4. Sideropore production under *in vitro* condition by six different fluorescent pseudomonads.

Fluorescent pseudomonad isolates	Siderophore production (µM benzoic acid/mL) Mean ± SD
MFP9	2.04 ± 0.13
CHFP23	11.55 ± 0.21
TFP16	9.87 ± 0.25
FPK7	6.98 ± 0.23
FPK8	15.20 ± 0.33
FPK10	13.9 ± 0.30

Average mean ± SD of three replicates, $P < 0.05$.

Assay for siderophore typing

The type of siderophore was determined by Csaky and Arnow assay, where isolates TFP16 and FPK8 has shown hydroxamate type of siderophore production that is formation of wine red colour in supernatant. Further spectrophotometric analysis of the culture, TFP16 and FPK8 in King's B medium showed a sharp peak between 420 to 450 nm (Figure 3a) and clearly indicated the presence of siderophores of ferrate hydroxamate nature.

Catecholate type of siderophore was determined by Arnow's test where the formation of yellow colour was observed in the supernatant of the isolates MFP9, CHFP23, FPK7, FPK10. A peak seen between 490 to 505 nm confirmed the presence of siderophores of catecholate nature (Figure 3b and 3c) (Ali and Vidhale, 2011). Evidence for the production of catecholate or hydroxamate siderophores by different bacterial species has been shown previously (Hohlneicher et al., 1995; Howard et al., 2000; Gull and Hafeez, 2012).

Siderophores usually form a stable, hexadentate, octahedral complex with Fe^{3+} preferentially compared to other naturally occurring abundant metal ions. Siderophores are usually classified by the ligands used to

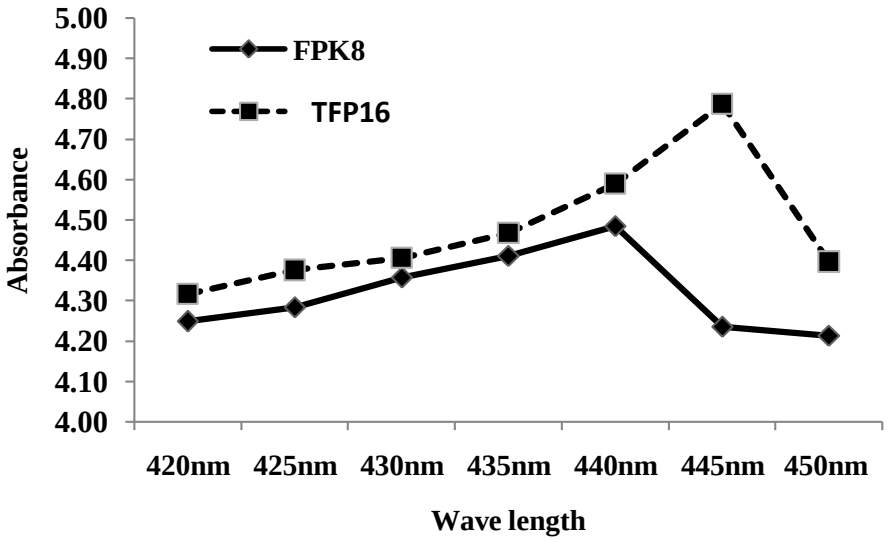

Figure 3a. Absorption characteristics FPK8 and TFP16 extract confirming hydroxamate nature of siderophores. Scanning of absorption characteristics were between 400-550 nm; a sharp peak between 420 and 450 nm confirmed hydroxymate nature of siderophore of FPK8 and TFP16.

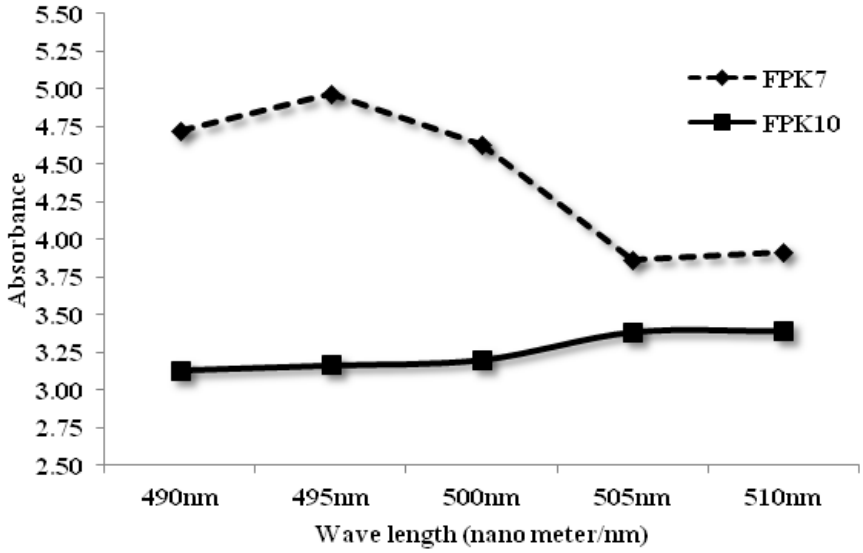

Figure 3b. Absorption characteristics FPK7 and FPK10 extract confirming catecholate nature of siderophores. Scanning of absorption characteristics were between 400-550 nm; a sharp peak between 490 and 510 nm confirmed catecholate nature of siderophore of FPK7 and FPK10.

chelate the ferric iron. The majors groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (ederivatives of citric acid) (Hofte, 1993).

Temporal variation of bacterial growth and siderophoregenesis

In the growth and siderophore production, as depicted in Figure 4a to 4f, an exponential phase from 6 to 48 h was observed regarding the isolates MFP9, CHFP23, TFP16 and FPK8; stationary phase was initiated after 48 h. In the case of the isolates FPK7 and FPK10, growth was ceased after 72 h. Threshold level of siderophore production was observed after 12 h of incubation. Syeed et al. (2005) also found similar result that siderophore production started after 12 h of incubation, while working

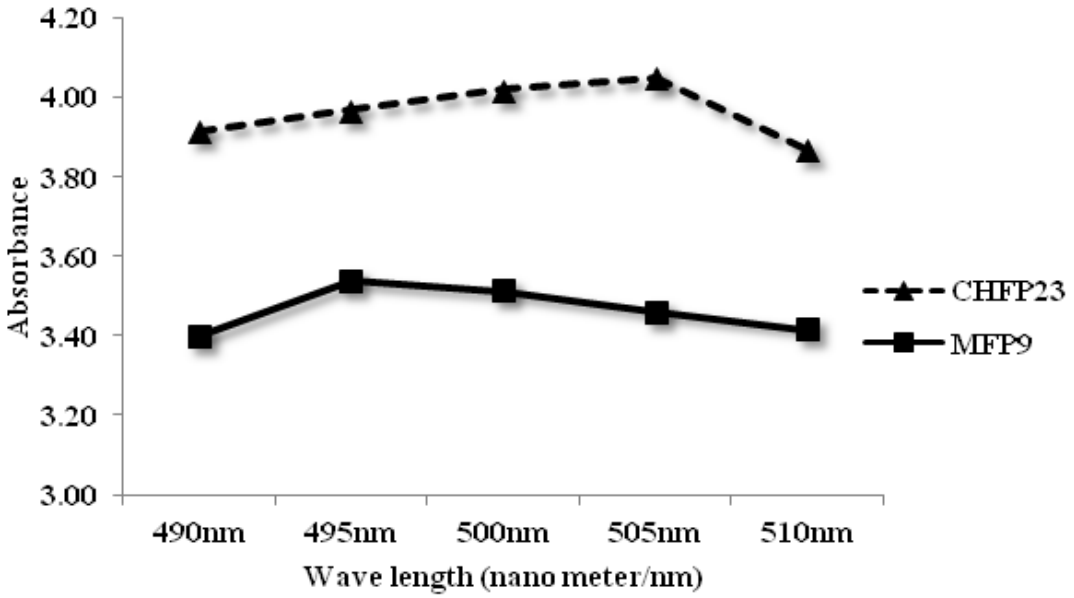

Figure 3c. Absorption characteristics MFP9 and CHFP23 extract confirming catecholate nature of siderophores. Scanning of absorption characteristics were between 400-550 nm; a sharp peak between 490 and 510 nm confirmed catecholate nature of siderophore of MFP9 and CHFP23.

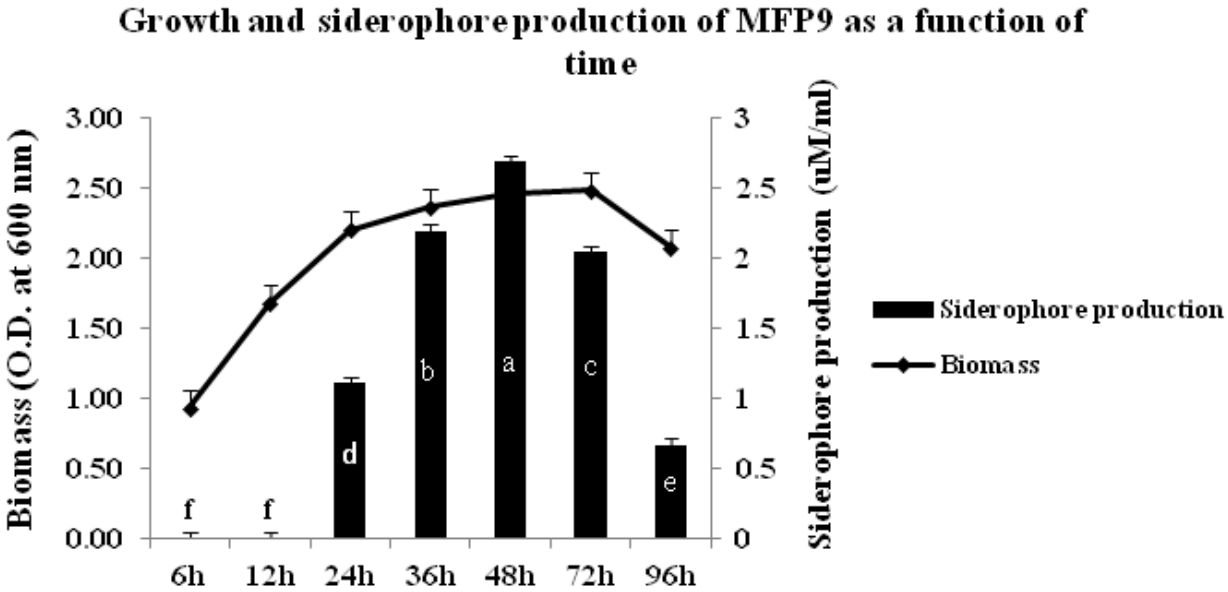

Figure 4a. Temporal growth measurement and siderophore production of MFP9 as a function of time. Vertical bar shows critical difference values.Values are mean of three replications. A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

with two fluorescent pseudomonads. For all the five isolates except MFP9, production of siderophore was increased up to 72 h and declined thereafter, for MFP9 it was after 48 h. Thus, maximum siderophore yield was observed at the end of log phase growth (Sharma and Johri, 2003).

Influence of iron on siderophoregenesis

In order to assess the influence of iron concentration on siderophore release of six different isolates, King's B media supplemented with different concentration of ferric ion was used. After 72 h of incubation, maximum side-

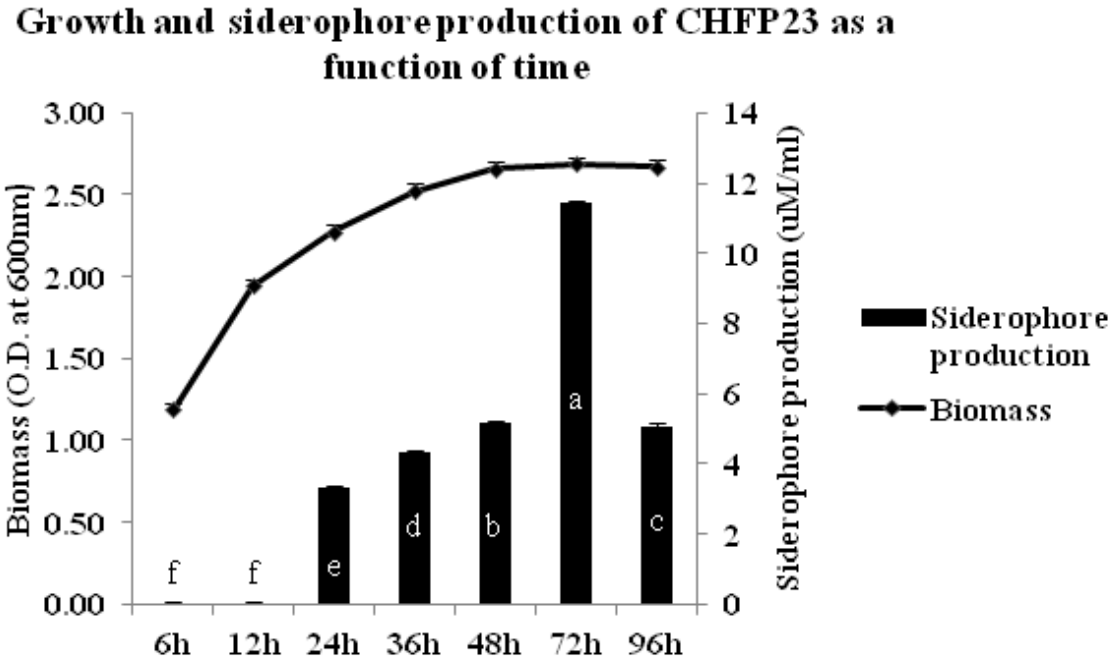

Figure 4b. Temporal growth measurement and siderophore production of CHFP23 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

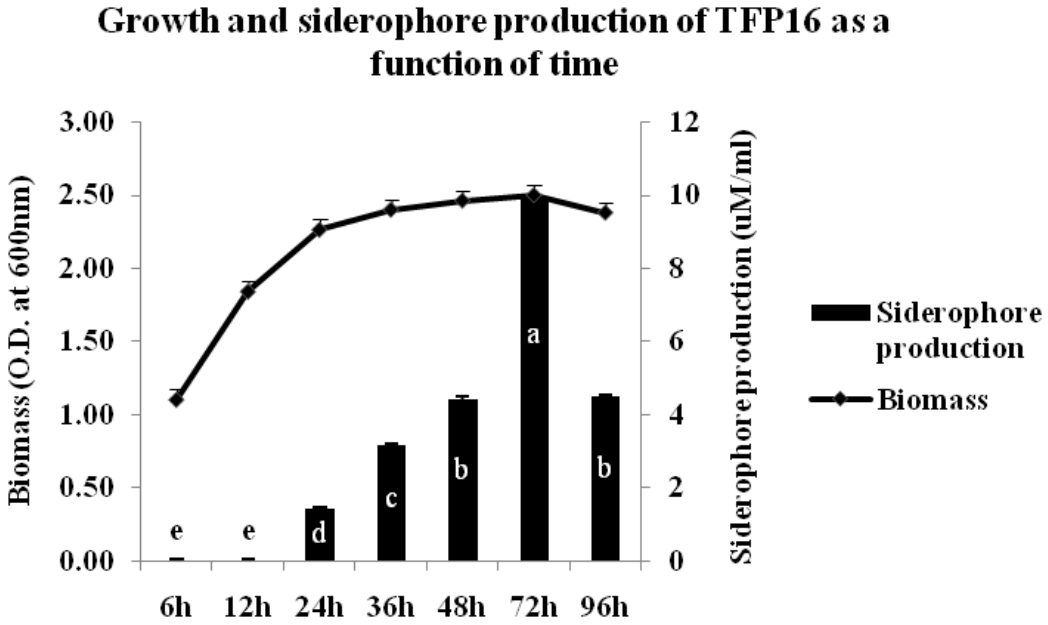

Figure 4c. Temporal growth measurement and siderophore production of TFP16 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

rophore productions for all the isolates under study were obtainable at 0.5 µM Fe^{3+} concentration and the lowest siderophore level was recorded at 10.0 µM Fe^{3+} (Table 5). Siderophores are iron-specific compounds which are synthesized and secreted under iron stress condition and for each isolate under study siderophore production was

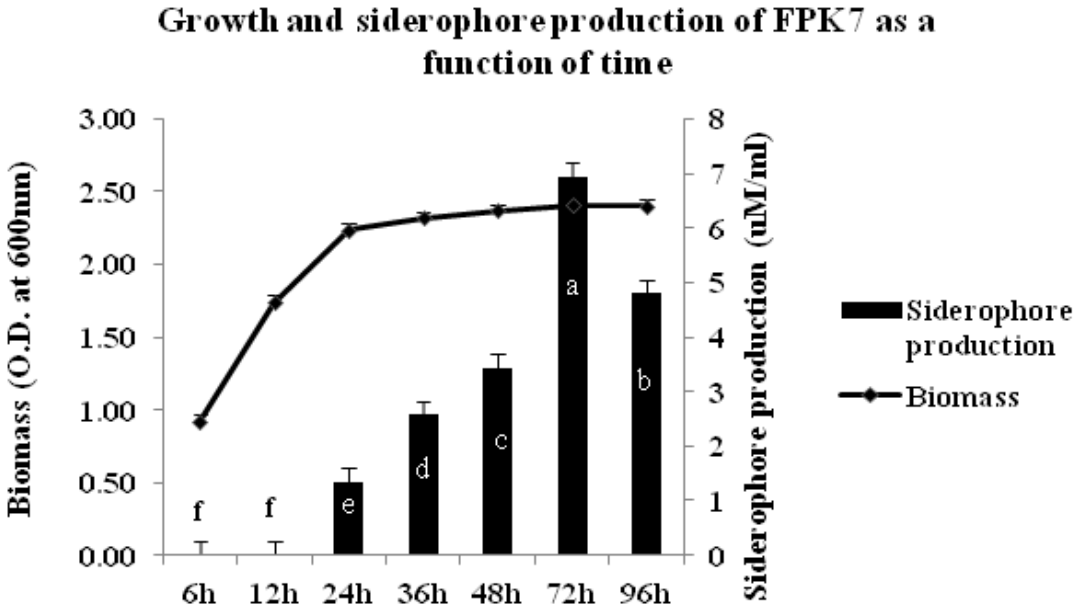

Figure 4d. Temporal growth measurement and siderophore production of FPK7 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

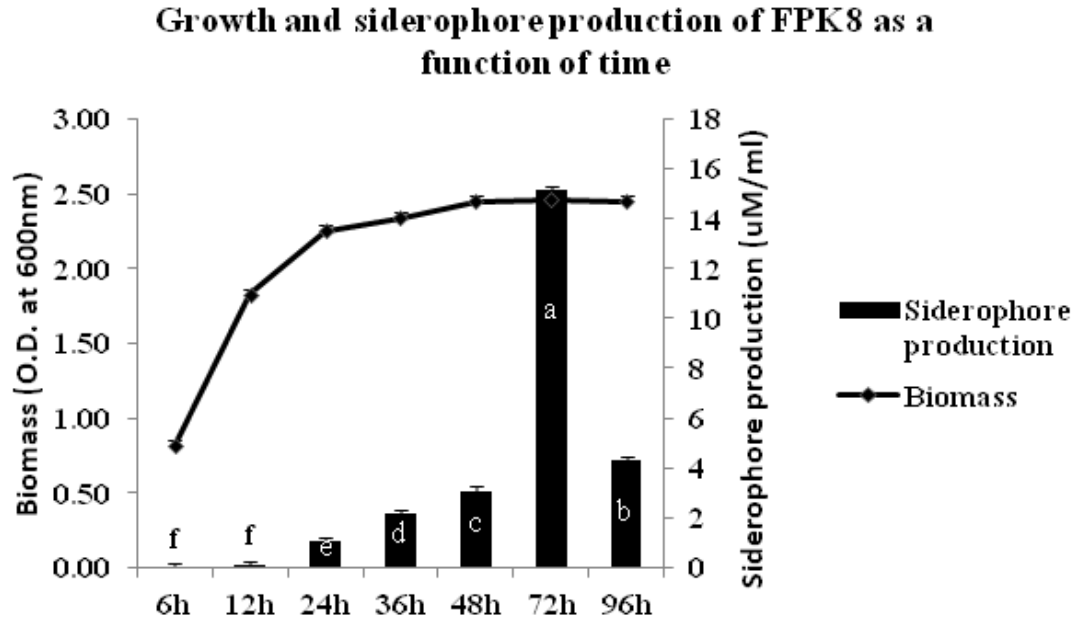

Figure 4e. Temporal growth measurement and siderophore production of FPK8 as a function of time. Vertical bar shows critical difference values. Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

gradually repressed with the increasing concentration of iron (0.5, 1, 1.5, 2.5, 5, 10µM $FeCl_3$) (Budzikiewicz, 1993; Rachid and Ahmed, 2005; Sayyed et al., 2005).

The universal repressor 'Fur' acts together with iron under iron rich conditions. When the intra-cellular environment is iron rich, the Fe^{2+}-Fur complex bind to promoter containing a 'Fur box' and represses transcription of the genes involving in iron uptake. Moreover,

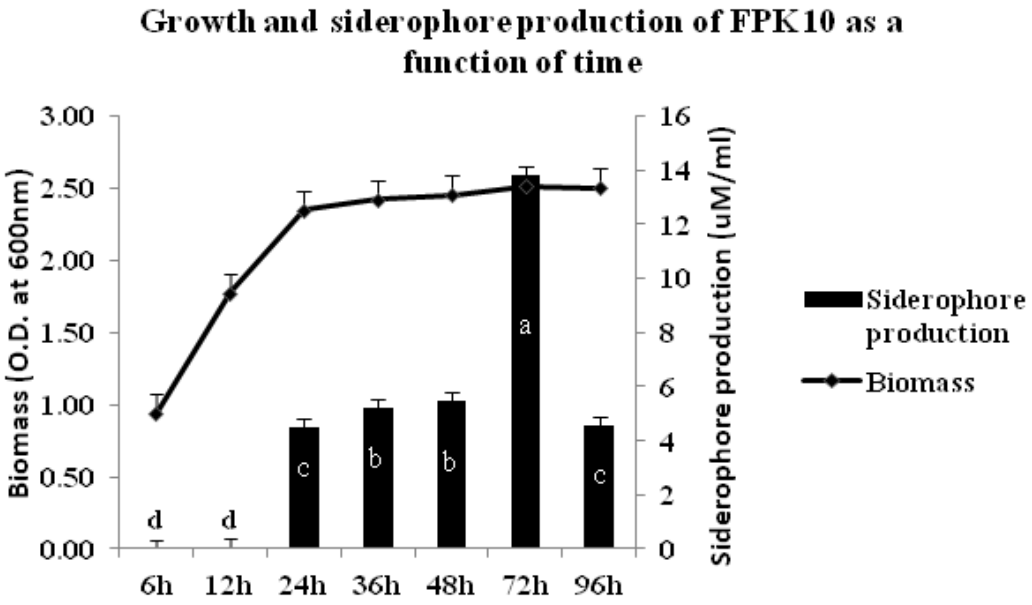

Figure 4f. Temporal growth measurement and siderophore production of FPK10 as a function of time. Vertical bar shows critical difference values.Values are mean of three replications .A common letter (letters on the bars) means they are not significantly different (p= 0.05) by DMRT.

Table 5. Influence of different ferric ion concentration on siderophoregenesis (µM benzoic acid/mL).

Fluorescent pseudomonad isolates	Siderophoregenesis (µM benzoic acid/mL)				
	0.5 µM $FeCl_3$	1.5 µM $FeCl_3$	2.5 µM $FeCl_3$	5 µM $FeCl_3$	10 µM $FeCl_3$
MFP9	0.53^{e}	0.20^{c}	0.00^{e}	0.00^{e}	0.00^{e}
CHFP23	6.81^{b}	5.92^{a}	5.71^{a}	4.48^{a}	2.62^{a}
TFP16	5.84^{c}	5.62^{a}	5.16^{b}	3.04^{c}	1.95^{b}
FPK7	4.76^{d}	4.49^{b}	3.73^{d}	3.02^{c}	1.60^{c}
FPK8	11.33^{a}	4.46^{b}	4.25^{c}	3.53^{b}	2.45^{a}
FPK10	5.18^{cd}	4.46^{b}	3.53^{d}	2.17^{d}	1.15^{d}
SEM (±)	0.216	0.198	0.104	0.115	0.080
CD (p = 0.05)	0.683	0.624	0.329	0.364	0.255

Values are mean of three replications. A common letter (on the superscript of each value) means they are not significantly different (p= 0.05) by DMRT.

there are several reports of *Pseudomonas aeruginosa* and *Vibrio anguillarum* regarding positive regulatory mechanisms of siderophore producing genes and iron uptake systems by a combination of positive regulatory proteins and cognate siderophore but the details are under study (Chen et al., 1996; Crosa, 1997; Heinrichs and Poole, 1996; Brandel et al*.,* 2012).

Invitro antagonistic effect on mycelial growth of phytopathogen under different iron limiting conditions

All the isolates were tested for *in vitro* siderophore mediated antagonism at different iron (Fe^{3+}) concentration against *R. solani* and *S. rolfsii*. All the six isolates showed percent inhibition, to some extent, at all the different iron concentrations. Although, the present study argued that percent inhibition and or zone of inhibition decreased with increasing iron concentration when all the other parameters such as growth temperature, incubation period, defined media compositions are constant (Kumar et al*.,* 2002; Sharma and Johri, 2003).

At 0.5 µM iron concentration, the percent inhibition of mycelial growth of *S .rolfsii* and *R. solani* for FPK8 were 71.1 and 73.3% respectively, which are the maximum among all the isolates, whereas, the same was 52.2% at 10 µM concentration of iron. The similar phenomenon is true with regard to the other isolates (Table 6a and b). It is apparent from the data that the siderophore mediated

Table 6a. Siderophore mediated *in vitro* antagonism against *S. rolfsii* at different iron concentration.

Fluorescent pseudomonad isolates	Different concentration of ferric ion									
	0.5 (µM)		1.5 (µM)		2.5 (µM)		5 (µM)		10 (µM)	
	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibiti (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)
MFP9	1^{c}	63.3^{d}	0.6^{b}	58.9^{c}	0.2^{d}	52.2^{e}	0.0^{d}	44.4^{e}	0.0^{b}	38.9^{e}
CHFP23	1.5^{a}	67.8^{c}	0.9^{a}	60.0^{c}	0.7^{a}	55.6^{c}	0.5^{a}	51.1^{c}	0.2^{a}	43.3^{c}
TFP16	1.2^{b}	57.8^{e}	1.0^{a}	55.6^{d}	0.4^{c}	53.3^{d}	0.2^{c}	46.7^{d}	0.0^{b}	41.1^{d}
FPK7	0.7^{d}	71.1^{a}	0.5^{b}	68.9^{a}	0.2^{d}	64.4^{a}	0.0^{d}	58.9^{a}	0.0^{b}	52.2^{a}
FPK8	0.6^{d}	68.9^{b}	0.5^{b}	66.7^{b}	0.5^{b}	61.1^{b}	0.3^{b}	56.7^{b}	0.2^{a}	50.0^{b}
FPK10	0.3^{e}	53.3^{f}	0.2^{c}	47.8^{e}	0.0^{e}	44.4^{f}	0.0^{d}	37.8^{f}	0.0^{b}	33.3^{f}
SEM (±)	0.057	0.254	0.054	0.409	0.019	0.043	0.011	0.136	0.017	0.107
CD (p = 0.05)	0.181	0.803	0.172	1.288	0.060	0.136	0.037	0.430	0.054	0.338

Values are mean of three replications .A common letter (on the superscript of each value) means they are not significantly different (p= 0.05) by DMRT.

Table 6b. Siderophore mediated *in vitro* antagonism against *R. solani* at different iron concentration.

Fluorescent pseudomonad isolates	Different concentration of ferric ion									
	0.5 (µM)		1.5 (µM)		2.5 (µM)		5 (µM)		10 (µM)	
	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)	Zone of inhibition (cm)	Inhibition (%)
MFP9	1.3^{b}	64.4^{e}	1.2^{b}	61.1^{e}	1.0^{a}	58.9^{e}	0.7^{b}	56.7^{d}	0.4^{b}	47.8^{c}
CHFP23	1.6^{a}	75.6^{a}	1.3^{a}	73.3^{a}	$+0.9^{a}$	71.1^{a}	0.7^{b}	67.8^{a}	0.5^{a}	52.2^{a}
TFP16	1.3^{b}	67.8^{d}	1.2^{b}	64.4^{d}	1.0^{a}	62.2^{d}	0.8^{a}	58.9^{c}	0.4^{b}	50.0^{b}
FPK8	0.8^{c}	73.3^{b}	0.6^{d}	72.2^{b}	0.4^{b}	65.6^{b}	0.2^{d}	60.0^{b}	0.0^{d}	52.2^{a}
FPK7	0.8^{c}	72.2^{c}	0.7^{c}	67.8^{c}	0.5^{b}	63.3^{c}	0.4^{c}	58.9^{c}	0.2^{c}	52.2^{a}
FPK10	0.4^{d}	55.6^{f}	0.2^{e}	54.4^{f}	0.0^{c}	51.1^{f}	0.0^{e}	43.3^{e}	0.0^{d}	34.4^{d}
SEM (±)	0.012	0.176	0.013	0.136	0.045	0.133	0.021	0.170	0.021	0.047
CD (p = 0.05)	0.039	0.556	0.042	0.430	0.141	0.421	0.067	0.536	0.068	0.148

Values are mean of three replications. A common letter (on the superscript of each value) means they are not significantly different (p= 0.05) by DMRT.

antagonism may be one of the important characters for the fluorescent pseudomonas under study.

Conclusion

Based on these results, it can be concluded that the production of different and potent antifungal hydroxymate and catecholate siderophore by fluorescent pseudomonads against *R. solani* and *S. rolfsii* are being reported and could be effective bio-control agent against these phytopathogens. Reduction of antagonism by the addition of iron indicated that siderophores were inhibitory to fungal growth and showed a significant effect. Though different mechanisms might be responsible for the inhibition of *R. solani* and *S. rolfsii* but siderophore production might be the main bio-control mechanism associated with the antagonistic potentiality of the rhizobacterial isolates of West Bengal.

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Full Length Research Paper

Validation of the acaricidal properties of materials used in ethno-veterinary control of cattle ticks

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Ticks are vectors of tick-borne diseases, cause teat damage and tick-worry. They are commonly controlled using conventional acaricides, which are expensive to the resource-limited farmers, making them to resort to alternative tick control materials. The objective of this study was to validate the acaricidal properties of various ethno-veterinary materials used by rural farmers in the Eastern Cape Province of South Africa. *In vitro* repellency and contact bio-assay models were carried out to determine the repellency and acaricidal properties of *Ptaeroxylon obliquum, Aloe ferox* Mill*, Lantana camara* L*, Tagetes minuta,* used engine oil and Jeyes fluid on *Rhipicephalus sanguineus* ticks. The optimum repellency of Jeyes fluid at concentrations of 76.8 and 100% lasted for 6 and 7 h, respectively. Tabard the reference product lasted for 4 h. *P. obliquum* (40%) repelled the ticks for 40 min. For the contact bio-assay, used engine oil, *T. minuta* oil (50%), Ektoban® and Jeyes fluid (76.8%) caused tick mortality of more than 86%. This study reveals that the materials rural farmers use to control ticks vary in their efficacy. Jeyes fluid and used engine oil have acaricidal effect as the conventional acaricides whereas extracts of *A. ferox*, *L. camara* and *T. minuta* are not.

Key words: Acaricides, ethno-veterinary materials, repellency, *Rhipicephalus sanguineus*

INTRODUCTION

Ticks are one of the major factors that contribute to animal health problems and exert the greatest limitation in cattle production, by causing serious debility, morbidity, mortality and production losses in cattle (Okello-Onen et al., 2004; Moyo and Masika, 2009). They also transmit pathogens such as *Anaplasma* spp., *Babesia* spp., *Theileria* spp. and *Ehrlichia* species (Bram, 1983; L'Hostis and Seegers, 2002). The common tick species in the Eastern Cape Province of South Africa are *Rhipicephalus appendiculatus, Rhipicephalus evertsi evertsi, Rhipicephalus (Boophilus) decoloratus* and *Rhipicephalus sanguineus* (Marufu et al., 2010).

Ticks are commonly controlled using conventional acaricides which are applied as dips or sprays and pour-on at various frequencies. Some of the conventional acaricides have repellency or acaricidial properties. Use of repellent acaricides for animal protection against ticks, constitute an important prophylactic component of tick-borne disease (TBD) management strategy (Dautel, 2004). Same applies to the contact acaricides which are chemical agents meant to kill ticks and are largely toxic through contact action (Boden and West, 1998). Some of the acaricides have long residual effect while others degrade rapidly after application (Brimecombe, 2006). Unfortunately, these chemicals are expensive, not readily available to the resources-limited farmers. In addition, the

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undegradable residues contained in conventional acaricides may pollute the products (milk and meat) and the environment, and ticks have developed resistance to them (Okello-Onen and Rutagwenda, 1997). This has caused farmers to turn to low cost alternatives such as ethno-veterinary materials namely used engine oil, Jeyes fluid, *Aloe ferox* Mill and *Ptaeroxylon obliquum* (Moyo and Masika, 2009; Moyo et al., 2009).

The alternative materials like used engine oil are highly complex mixture, containing compounds distilled from petroleum, for example aliphatic hydrocarbons, aromatic hydrocarbons, polycyclic aromatic hydrocarbons [PAHs]), as well as metals such as aluminium, chromium, lead, manganese (Delistraty and Stone**,** 2007). Also, the Jeyes fluid a commercial disinfectant which is registered for use on non-living materials contains carbolic acid 13% m/m identified as phenol (6H5OH) and sodium hydroxide (1%) (Chem. Alert Report, 2005). However, some of these alternatives like Jeyes fluid and used engine oil are equal or more toxic than the conventional acaricides and are not the best option to be used in tick control in livestock (Aitken and Barrett, 2007; Delistraty and Stone, 2007).

In Zimbabwe for example, ethno-veterinary medicine is gaining recognition at the expense of conventional drugs especially because of its greater accessibility, lower costs and apparent effectiveness (Mwale et al., 2005) and little work on validation have been done. Many small-scale farmers are known to use ethno-veterinary practices for the control of ticks (Njoroge and Bussmann, 2006).

The purpose of this study was to validate the acaricidal properties of *P. obliquum, Aloe ferox, Lantana camara, Tagetes minuta*, used engine oil and Jeyes fluid on *R. sanguineus* ticks.

MATERIALS AND METHODS

Plant material collection

The leaves of *A. ferox* Mill, Voucher number BM01-037/2007, *L. camara* L Voucher No. BM01-039/2007, *T. minuta* L Voucher No. BM01-040/2007 and the bark of *P. obliquum* (Thunb) Radlk Voucher No. BM01-038/2007 were collected before flowering within Amathole District Muncipality (30°15′ 15" S and 22°15′ 36" E), South Africa.

Preparation of the plant materials

Aqueous extraction of dried plant material

Fresh plant material (leaves of *L. camara, T. minuta*, *A. ferox* and the fresh bark of *P. obliquum*) were collected, air dried under shade for six weeks and then milled into powder using a hammer mill with 1 mm pore size sieve according to the farmers' description. 100 g of the respective plant materials were mixed with 1000 ml of distilled water and soaked overnight. The extracts were filtered using Whatman No.1 filter paper. The filtrate was then lyophilized using a freeze drying system Xerotec (model Cd 052, Kenmore international, Italy) for 72 h, yielding 10, 9, 11 and 12 g of extracts of *L. camara, T. minuta*, *A. ferox and P. obliquum*, respectively. The resulting extracts was stored in capped bottles and kept in the refrigerator until use. The extracts were reconstituted using distilled water to make different concentrations of 10, 20 and 30% (w/v).

Aqueous extraction of fresh plant material

Fresh leaves of *L. camara, T. minuta* and *A. ferox* and the fresh bark of *P. obliquum* were collected and thoroughly washed using distilled water. Plant material of 40, 60 and 80 g were placed in 200 ml of distilled water and macerated for 1 min using an electric Sunbeam Deluxe glass blender model SGB-150, Johannesburg, South Africa.

Generally, the farmers crush the plant materials using mortar and pestle. The mixture was stored at room temperature overnight and later strained using a muslin cloth. The concentration percentages of extracts were determined using weight per volume (Jayasinghe, 1975) to obtain a 20, 30 and 40% (w/v) extract. The plant extracts were prepared as per information given by the farmers (Moyo and Masika, 2009).

Extraction of oil

Quantities (200 g) of leaves for *L. camara, A. ferox, T. minuta* and the bark of *P. obliquum* were mixed with 5 L of distilled water separately subjected to hydro distillation for 3 h using a clevenger-type apparatus (Soffiera vetro, Sassari, Italy). With the exception of *T. minuta*, the others yielded negligible quantities of oil. The Tagetes oil was diluted to concentrations of 3.125, 6.25, 12.5, 25, 50 and 100% using olive oil, to find out which concentration has the acaricidal activity.

Extraction of aloe leaf exudates

The aloe leaf exudates were obtained by cutting the aloe leaves and the exudates was collected, air dried and stored in bottles. It was reconstituted to the following concentrations: 1.8, 3.5, 7.5 and 15% using distilled water. Different extraction methods and concentrations of materials were used to assist in the screening of materials having acaricidal properties.

Preparation of non-plant materials

Jeyes fluid (Adcock Ingram, Bryanston, South Africa), a household disinfectant was diluted using distilled water to make the following concentrations: 0.6, 1.2, 2.4, 4.8, 9.6, 19.2, 38.4, 76.8 and 100%. Used engine oil was used undiluted. A commercial acaricides, Ektoban® (cymiazol 17.5% w/v and cypermethrin 2.5% w/v - Novartis, Johannesburg, South Africa) registered for the control of ticks (Swan, 2001) was also used. A standard insect repellent material, Tabard (35% diethyloluamide, Acorn (Pty) Ltd, strubens Valley, South Africa) which is effective against fleas, ticks, flies and gnats (Acorn South Africa (Pty) Ltd, 2009, personal communication) was used as a positive control.

Ticks

Adult and nymph stages of *R. sanguineus* were obtained from Clinvet International, Bloemfontein, South Africa. This tick species was selected for the study because it affects both cattle and dogs. In addition, it was the only tick species that was cultured having same age and from the same environment. The ticks were held in small transport vials supported with moist Whatman filter paper and fresh green grass. They were kept at 75% relative humidity and a temperature of 25 ±1°C, to produce an environment conducive for the survival of ticks (Thorsell et al., 2005).

In vitro repellency bioassay

The repellency method described by Thorsell et al. (2005) was used in this bioassay. The nymphal stage of *R. sanguineus* tick species was used to test the repellency properties of ethno-veterinary materials (Chungsamarnyart et al., 2003). Two filter papers (Whatman No. 1) were placed inside a Petri-dish with an inner diameter of 9.5 cm. Tabard (diethyltoluamide 350 mg) an insect repellent was used as positive control while distilled water was used as negative control. Aliquots of 0.5 ml of the test solutions were applied along the periphery of the filter papers. The filter papers were then air dried for 2 min. Each test was replicated three times.

Six nymphs were placed at the centre of each of the treated filter papers and their movement with regards to avoiding the treated area was observed. If the nymph continued its motion beyond the periphery of the treated area within 5 min, the tick was indicated as non-repelled; conversely, if the nymph reversed its direction before reaching the periphery of the treated area the tick was considered as repelled. The Petri dishes were uncovered and exposed to the air from the start to the end of the experiment. The test was repeated after 0.33, 0.67, 1, 2, 3, 4, 5, 6, 7, 8 and 9 h after treatment. At each occasion, the number of nymphs avoiding the treated area was recorded. The repellency was expressed as number of nymphs avoiding treated area to the total number of nymphs at each occasion. Thus, 6 nymphs avoiding the treated area out of a total of 6 is recorded as 100% repellency. The repellency was calculated according to Thorsell et al. (2005) as follows:

$$R = \frac{a}{n} \times 100$$

Where R is the repellency; a = the number of nymphs avoiding the treated area; and n = the total number of nymphs put in the centre of filter paper at each occasion.

Contact bioassay on ticks

The dipping method was used for *in vitro* bioassay (Pirali-Kheirabadi et al., 2007). Adult *R. sanguineus* ticks were divided into groups of five and each group was immersed in a specific concentration of test samples ranging from 10, 15, 20, 30 to 40% for one minute. Distilled water and acaricide (Ektoban®) were used as negative and positive control, respectively. After dipping in the respective materials, each group was placed into separate Petri dishes containing moist Whatman No. 1 filter papers measuring 62.63 cm^2, with pieces of green grass to provide an environment conducive for tick survival (Thorsell et al., 2005). The tick samples were incubated for 7 days at 25°C and 75% relative humidity in the dark as described by Pirali-Kheirabadi et al. (2007) with slight modifications. Each treatment was replicated three times. The Petri dishes were examined on an hourly basis for the first 6 h after treatment, and thereafter every 24 h in the morning; to count and remove the dead ticks (Pamo et al., 2005). The tick mortality rate was calculated according to Abott (1925) and Chungsamarnyart et al. (2003) as follows:

$$\text{Corrected mortality (\%)} = \left(1 - \frac{T}{C}\right) \times 100$$

Where: T is the number of ticks remaining alive after treatment; C is the number of ticks remaining alive in the control group.

Ticks were considered alive if they exhibited normal behavior (movement looking for the host) when breathed upon or physically stimulated with a wooden stick. For each time point, if ticks were incapable of moving, maintaining a normal posture, leg coordination, being upright themselves, or showing any sign of life, they were considered moribund or dead (Panella et al., 2005).

Statistical analysis

In vitro repellency bioassay

The percentage tick repellency was calculated according to Thorsell et al. (2005). Mean repellency for all materials used was calculated.

Contact bio-assay

The following model was used to analyze tick mortality caused by treatment materials.

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where Y_{ij} = response effect (mortality) due to treatment; μ = overall mean (constant); T_i = effect due to treatment ($_i$ = 1, 2, 3… and 32); E_{ij} = residual error.

The collected data was then analyzed using PROC GLM for repeated measures (SAS, 2003). P-values < 0.05 were regarded as significant. Duncan test was used to compare differences between treatment means.

RESULTS

In vitro repellency bioassay

With the exception of *P. obliquum*, the other plant extracts did not show any tick repellency effect. Only *P. obliquum* (40%) and Jeyes fluid (9.6 to 100% concentration) showed repellency as shown in Table 1. The repellency duration was shorter for *P. obliquum* (40 min) than Jeyes fluid >9.6% (1-8 h). The Jeyes fluid at 76.8 and 100% concentration was effective (repellent) for 7 and 8 h, while the positive control, Tabard was effective for 4 h after application. Extracts of *L. camara* (20 and 40%), *A. ferox* (20 and 40%), *T. minuta* (20 and 40%), *T. minuta* oil, used engine oil, olive oil and *A. ferox* leaf exudates (1.8, 3.5, 7.5 and 15%) showed no repellency The repellency of Jeyes fluid was effective from a concentration of 2.4 to 100%.

Contact bio-assay

The plant extracts did not yield any acaricidal properties. Used engine oil, Ektoban®, *T. minuta* oil (25 and 50%) and Jeyes fluid (19.2, 38.4, 76.8, and 100%) showed variable acaricidal efficacy with the following mortalities: 93, 87, 13, 100, 26.7, 66.7, 86.6 and 100%, respectively within the first 24 h after application as shown in Table 2. *A. ferox* leaf exudates immobilized the ticks for the first 12 h and later they became active.

DISCUSSION

In all the experiments, it was clear that the nymphs displayed

Table 1. Tick repelling activity of *P. obliquum* and Jeyes fluid at different concentrations.

Material	Tick repellency (%)										
	20 min	40 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h	9 h
P. obliquum (40%)	100	94	0	0	0	0	0	0	0	0	0
Jeyes fluid (9.6%)	100	100	72	0	0	0	0	0	0	0	0
Jeyes fluid (19.2%)	100	100	100	94	22	0	0	0	0	0	0
Jeyes fluid (38.4%)	100	100	100	100	78	22	0	0	0	0	0
Jeyes fluid (76.8%)	100	100	100	100	100	100	100	89	33	0	0
Jeyes fluid (100%)	100	100	100	100	100	100	100	100	83	28	0
Tabard (35%)	100	100	100	100	100	94	56	6	0	0	0

Table 2. Least square means for acaricidal effect of some ethno-veterinary remedies.

Material	Tick mortality (%)													Total tick mortality (%)
	1 h	2 h	3 h	4 h	5 h	6 h	1 day	2 days	3 days	4 days	5 days	6 days	7 days	
Used engine oil	73	0	0	13	7	0	93	0	0	0	0	0	7	100[a]
T. minuta oil (50%)	60	40	-	-	-	-	100	-	-	-	-	-	-	100[a]
T. minuta oil (100%)	100	-	-	-	-	-	100	-	-	-	-	-	-	100[a]
Jeyes fluid (38.4%)	6.7	60	0	0	0	0	66.7	0	0	0	0	0	0	66.7[c]
Jeyes fluid (76.8%)	73.7	13.3	0	0	0	0	86.6	0	0	0	0	0	0	86.6[b]
Jeyes fluid (100%)	100	-	-	-	-	-	100	-	-	-	-	-	-	100[a]
Ektoban	87	0	0	0	0	0	87	13	-	-	-	-	-	100[a]

[a,b,c]Means in the column with different superscript letters differ significantly, $p<0.05$.

their natural host-seeking behavior during the tests. They moved around the filter paper and even crossed the treated areas for the extracts that did not show repellency.

The extracts of *P. obliquum* at 40% concentration had a short-lasting protection that provided 94% repellency at 40 min post-application against the nymph. This short repellency period may not be adequate to protect livestock against ticks. Tabard stick, our positive control, provided greater than 94% repellency for 4 h which was greater than the repellency period of *P. obliquum.* The bark of *P. obliquum* has been reported to contain saptaeroxylon (an acid saponin), pyrogall, resins and an alkaloid (Archer and Reynolds, 2001) with resins reported to have repellency activities (Pontes et al., 2007).

It is speculated that the resin oil in the *P. obliquum* was in small quantities which made the repellency to last for a short period of time or it was volatile in nature making it to lose its repellency. Therefore, further research is necessary to find ways of improving the duration period of repellency. The results of the contact bioassay are in contrast with those of Archer and Reynolds (2001), who indicated that the powder of the bark added to water kills cattle ticks. The variation could be attributed to the tick species and the age of the tree from where the bark was collected and growth environment which can influence the amount of compounds in the plant. The results obtained in this study indicate that essential oil of *T. minuta* had no repellency properties, however, it differs from the studies of Nchu et al. (2012) which showed some tick repellency against *Hyalomma rufipes*. This could

be attributed to differences in tick species used in the study.

Jeyes fluid showed some acaricidal efficacy both through contact and repellency in the study which compared well with the positive control, Ektoban® acaricide. The active ingredient of Jeyes fluid (carbolic acid) was once used as carbolic dip to control ticks more than 50 years ago (Malesela, 2004). Moreover, the carbolic acid is poisonous to tissues especially when applied directly to muscle and nerves, causing paralysis to the nerves which later paralysis the heart (Henriette, 2008) hence death of organisms.

However, the veterinarians do not recommend its use because carbolic dip has very narrow safety margins (Malesela, 2004; Aitken and Barrett, 2007). Carbolic dip caused some poisoning to animals dipped (Linklater et al., 1982; Aitken and Barrett, 2007). Moreso, its exposure in the animal skin have pathological effect (Chem Alert Report, 2005). The above reasons could have contributed to its withdrawal from the list of registered acaricides. In addition, Jeyes fluid is toxic to aquatic organisms, it alters the water pH (Irwin et al., 1997).

Jeyes fluid is locally available in shops and farmers maintain that it was cheaper as compared to the conventional acaricides and also kill ticks as reported by Malesela (2004) and Moyo et al. (2009). The residual effect of Jeyes fluid to consumers is not known. Therefore, its use should be avoided or used with caution and more research needs to be done on its toxicity.

The efficacy of used engine oil through contact bioassay compared well with the positive control conventional registered, Ektoban® acaricide in agreement with Moyo et al. (2009). This was in contrast with the field efficacy findings by Dreyer et al. (1998) where it had an average efficacy of 38.1%. The variation could be due to tick species or the field condition. The high mortality caused by used engine oil is ascribed to its action as a physical acaricide clogging the spiracles and causing the tick to suffocate (Dreyer et al., 1998). Therefore, direct contact between the oil and the tick is necessary for effective result (mortality). Mbati et al. (2002) and Masika et al. (1997) have documented the wide use of used engine oil. However, no validation was done. The toxic components in it, could be the ones that caused the death of the ticks.

The disadvantage of using used engine oil is that it has toxic components such as lead chromium, copper and zinc which have residual effect on plant and animal tissue making them unsafe for human consumption as cited by Moyo and Masika (2009). Used engine oil can contaminate the environment and water bodies resulting in the death of aquatic and soil microbes (Delistraty and Stone, 2007). This aspect needs investigation since little/no work has been done in this regard. However, many farmers in the Eastern Cape Province depend on government pensions (Dreyer, 1997), they cannot afford to buy conventional acaricides as such, they resort to using used engine oil to control ticks on their cattle.

The efficacy of *T. minuta* oil was comparable to that of the reference conventional acaricide, Ektoban®, which is very effective against ticks. This makes *T. minuta* oil an alternative acaricide that can be used by farmers. However, the cost of the oil may be prohibitive too. The acaricidal effect could be attributed to the compound terpens contained in *T. minuta* oil, which has been reported to have aphicidal and insecticidal properties (Sarin, 2004; Seyoun et al., 2007; Tomova et al., 2005). The effect of this oil on ticks, in this study concurs with its efficacy on other insects. The recommended concentration of *T. minuta* oil to be used as an acaricide could be 50% level. This is more economic than the 100% concentration which uses more essential oil.

Use of boiled *T. minuta* leaves kill ticks (Njoroge and Bussmann, 2006), as such farmers in the study area could benefit from boiling the leaves which is cheaper than distilling oil from the plants. The other plant extracts exhibited variable results ranging from no effect to lower mortality. Therefore, they cannot be used as alternative acaricides in tick control.

The extracts of *L. camara* at 40% concentration had a tick mortality of 27%, which was inadequate for reducing tick burdens in livestock. Our findings however are in contrast with Okello-Onen et al. (2004) and Moyo et al. (2009) where its efficacy was 96.6 and 58% for all the tick species, respectively. The difference in the observations could be due to difference in the environment where the plant was collected and age of the plant. This affects the chemical composition of the plant. Also, this variation could be ascribed to the fact that acaricidal efficacy depends on the tick species.

The results of this study have revealed that the materials rural farmers use as acaricides vary in their efficacy to control ticks. Jeyes fluid and used engine oil are as effective as the conventional acaricides whereas others are not effective at all. Jeyes fluid (38.4, 76.8 and 100%), *T. minuta* oil (50%) and used engine oil had the most acaricidal properties. However, they also need to be validated in *in vivo* experiments. Despite being effective, used engine oil and Jeyes fluid have potential toxic effects on animals and are also environmental contaminants.

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Full Length Research Paper

Effect of different ferric fertilizers on planting *Morchella conica* fruiting yields and analyses of the microflora and bioactivities of its grown soil

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The aims of this study were to determine whether six ferric fertilizers influence the development of fruit bodies of *Morchella conica* identified by analysis of the internal transcribed spacer (ITS), its grown soil microflora and enzymatic activities. We successfully cultivated one kind of *Morchella* in the wheat planting field, its partial ITS rDNA in the present study was identified as *M. conica* by GenBank and Morchella MLST database as a reference. Six ferric fertilizers were applied to the planting area. Four soil enzymatic activities (including soil urease, polyphenol oxidase, invertase, and protease) as well as soil microflora showed significant differences in the development stages of *M. conica* especially during the primordium differentiation stage. Results suggest that ferric fertilizer not only influenced the *Morchella* grown soil enzymatic activities and microorganisms, but also increased the *Morchella* yields while such effects differ from the species of the ferric fertilizer, especially groups with ammonium ferric sulfate [$NH_4Fe(SO_4)_2.12H_2O$], Fe·EDDHA and Tongfeng nutrients. So, this work highlights the importance of further attempts to resolve important aspects of the morel commercial cultivation regarding optimizing nutrition plan and figuring out the relationships between genotypes and their fertilities of fruiting body.

Key words: *Morchella conica*, identification, ferric fertilizer, microflora, enzyme activity.

INTRODUCTION

In the world's 1.5 million estimated fungi species (Hawksworth, 2004), few genera are as synonymous with epicurean cuisine as true morels (Du et al., 2012b), highly prized for their medicinal and nutritional qualities (Kanwal and Reddy, 2012). Fruiting bodies of morel fungi (*Morchella* spp., phylum Ascomycota) are highly restricted to temperate regions of the Northern Hemisphere in a few weeks each spring (O'Donnell et al., 2011). Due to the increasing popularity and commercial demand, wild morels are harvested commercially and exported extensively from China, India,Turkey, Mexico, and the United States (Pilz et al., 2007; Taskin et al., 2010).

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Abbreviations: **ITS**, Internal transcribed spacer; **CTAB**, hexadecyltrimethyl-ammonium bromide; **BLAST**, Basic Allignment Search Tool; **MLST**, multilocus sequence typing; **BS**, bootstrap; **PDA**, potato dextrose agar; **NCBI**, National Center for Biotechnology Information; **DGGE**, denaturing gradient gel electrophoresis.

The first successfully growing morel fruit body under controlled condition was carried out by cultivating in a specialized indoor facility using patented technology within the US (Ower et al., 1986), included outdoors in Yunnan Province of China (Zhao et al., 2009).

Morels' very short harvesting season and high price have encouraged researchers to study the biology and ecology of the species (Goldway et al., 2000). Many researchers have attempted to raise morels in controlled conditions; nevertheless, ascoscarps reaching the market are mostly collected from the wild (Ower et al., 1986; Goldway et al., 2000; Masaphy, 2005; Guler and Ozkaya, 2009; Masaphy, 2010). Their modes of reproduction, biology, nutritional sources, life cycle, are unusual and complex (David et al., 2007). Recently, a remarkable finding is that the majority of the Esculenta clade appeared to be adapted to mixed temperate deciduous hardwood forests with the Elata clade's preference to an evergreen coniferous biome (Du et al., 2012a; Donoghue, 2008). By all odds, it is important showing the morels growing conditions.

Previous reports are almost focused on their mycelial fermention or officinal functions, little on their cropping stages investigation. The mating systems of *Morchella* species are unknown (Buscot, 1993), and whether heterokaryosis (mating of two compatible strains) followed by meiosis occurs in natural populations of morels is also unknown (Volk and Leonard, 1989, 1990). Attention has focused on antioxidant activities of produced compounds, content of phenolics and flavonoids, autofluorescence and various metals (Gursoy et al., 2009; Kalač 2010). Many studies have demonstrated that minerals and heavy metals play an important role in the metabolic processes, during the growth and development of mushrooms in appreciable concentration (Claudia et al., 2010) and wild-growing mushrooms can accumulate great concentrations of toxic metallic elements and metalloids such as mercury, cadmium, lead, copper or arsenic and zinc (Vetter, 2004; Nevcihan et al., 2009; Zhu Fangkun et al., 2011). Reports pointed out that the accumulation of metals in fungi found to be affected by element environment such as organic matter amount, temperature, humidity, pH, metal concentrations in soil and fungal factors such as species of microfungi, morphological part of fruiting body, development stages and age of mycelium, biochemical composition, and interval between the fructifications affect metal accumulation in macrofungi (Mendil et al., 2005; Nevcihan et al., 2009).

It has also been shown that Fe was abundant in seven *Morchella* species, follow by calcium and magnesium, respectively (Nevcihan et al., 2009). Iron can stimulate lipid peroxidation by the fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Halliwell, 1991). As the most effective pro-oxidants, ferrous ions are commonly found in food systems (Yamaguchi et al., 1998).

Based on our previous study, we successfully cultivated *Morchella* in the wheat planting field. Up to now, the formation of fruit-bodies of morchella is still mysterious and their commercial production is not mature. Commercially cultivation of morel has also been confronting with series of difficulties. Many triggers for fruiting appear to differ by species to some extent. We still do not know which vital factor determines that the transformation from sclerotium of *Morchella* to primordium of fruit bodies, such as soil microorgansiam or microelements or other compounds, even the differences existing in genomices between fertile and infertile morchella strains.

In the present paper, our aims are to identify the morels using internal transcribed spacer (ITS) data with *Morchella* MLST as a reference and the effect of the different ferrous treatments on soil characteristics and the morel yields, in order to determine the possibility of optimal morels growing conditions.

MATERIALS AND METHODS

Strains and chemical agents

pMD18-T Vector Ligation Kit, DL2000 DNA Marker was purchased from TaKaRa Technology (Dalian) Co., Ltd. DNA Gel Extract Kit was purchased from MBI Fermentas. *Morchella conica* strain Mc-5 (commercially cultivated for five years), *Escherichia coli* DH5α and BL21 (DE3) strains were preserved in author's Laboratory. All other reagents except the noted agents used in present study were of analytical grade.

Molecular identification of *Morchella* strain and its sclerotia observation by scanning electron microscopy (SEM)

Total genomic DNA of *M. conica* was followed by a hexadecyl -trimethyl-ammonium bromide (CTAB) protocol (O'Donnell et al., 1997; Taşkin et al., 2010) when the mycelium was freeze-dried before the CTAB extraction step. The internal transcribed spacer (ITS) rDNA sequence was amplified using the total DNA as template while the primer pair of ITS1 and ITS4 was used as the primer pair, upstream primer (ITS1: 5'-TCCCTTACTCTTCTAACCTCTCCTCT-3') and downstream primer (ITS 4: 5'-TCCTCCC TCTTATTCTATATCTC-3'). Polymerase chain reaction (PCR) products were purified with the DNA Extraction Kit (MBI Ferments). The rDNA fragment was T-A cloned into the pMD18-T vector (Takara Shuzo Co., Ltd., Dalian, China) and propagated in *E. coli* (strain DH5α).

The positive clone was screened by Clone-PCR method (Gussow and Clackson, 1989; Sathe et al., 1991). The ITS sequences of the isolates was determined by Shanghai Jikang Biotech Company (Shanghai, China), then used as templates to search for homologous sequences in GenBank using the Basic Allignment Search Tool (BLAST) and Morchella MLST database as a reference (Robert et al., 2011). The obtained sequences were compared one to another by the computer-assisted method of Higgins and Sharp (1988). The phylogenetic relationships within the Morchellaceae using a aligned nucleotide positions in Paup via one thousand Maximum parsimony bootstrap (BS).

Experimental strain of *M. conica* Mc5 was grown in potato dextrose agar (PDA) and its sclerotia was harvested and fixed for 2 h at 4°C by using 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. Secondary fixation was performed in 1% osmium tetroxide in dH_2O after the samples were washed three times with 0.1 M cacodylate buffer (pH 7.4), and were then treated with 1% (w/v) osmium tetroxide, washed with 5% (w/v) sucrose in cacodylate buffer, and subsequently dehydrated in a graded ethanol series (from 30 to 100%). The samples were examined on a JEOL JSM-7500F Scanning Electron Microscopy after the works for critical point drying and gold coating were successfully carried out and the images were recorded.

Cultivation Experimental groups sprayed with ferric fertilizers

First fruit bodies pieces collected for pure *M. conica* culture isolation, sprayed with 70 % ethanol, were grown well in the PDA (potato 20 g/L, glucose 2g/L, agar 2g/L) test-tubes, then sub-culture of isolates were removed in another new sterilized medium which consists of wheat 70%, vermiculite 5%, pure soil 15% and chaff 10%, natural pH value and cultured under 15-18°C since mid-October for over 15 days, when morel sclerotia were formed well.

We choose Longxing Town (located in Chongzhou, Sichuan Province, China) where hardwood trees grow naturally and soil is sandy loam or humus without much clay. The climate is subtropical with annual precipitation and average temperature of 1012 mm and 17.8°C. Each section was 10×0.6 m^2. Each treatment was established with three replicates. Seeding of *Morchella* spawn was carried out during early November and about 1.5 bottle (750 mL/bottle) spawn were used per square meter (m^2) when the planting field tamped and leveled up firmly. About twenty days later after seeding in December, six experimental groups, including Iron II ethylenediamine di(*o*-hydroxyphenylacetic) acid (abbreviated as Fe·EDDHA, provided by Sichuan Tongfeng Science and Technology Co. Ltd, Chengdu, China), ferrous sulfate ($FeSO_4.7H_2O$), ferric sulfate [$Fe_2(SO4)_3.9H_2O$], ammonium ferric sulfate [$NH_4Fe(SO_4)_2.12H_2O$], citrate ferric, Tongfeng nutrition agents (abbreviated as nutrients, also provided by Sichuan Tongfeng Science and Technology Co. Ltd, Chengdu, China) with six trace elements (including with Fe, Zn, Mn, Mo, B, Cu, and its ferric content was about 2%), were separately sprayed onto the soil sampling districts grown with *M. conica*, and all the nutrition concentration of different ferric fertilizers was 100 mg/m^2 when *Morchella* mycelium grown well in the field before sclerotia formation, while the same volume of distilled water was sprayed as the control. Primordia formation started as temperatures increase above 10°C (a range between 6-15°C), lasting for one month since late January. The fruiting bodies appear approximately in the early February, obviously influenced by the soil temperature, and the fruiting season is no better than 20 days, compared with wild morels in ten days in other reports (Mihail et al., 2007; Wurtz et al., 2005).

Enumeration of major soil microbial population groups

Soil samples were randomly collected aseptically in ultra-violet (UV) sterilized plastic bags from each groups to a depth of 10~30 cm during sclerotia formation, primordia formation and fruit-body growth separately in late November, late January and early February. The soil samples were kept on ice during transport to the laboratory and stored at 4°C before microbial enumeration. The enumeration of the soil microflora was done by the dilution plate method (Nair and Subba-Rao, 1977). Soil sampling (10 g) was done from three replicated sites and emulsified in 90 mL sterilized water, aseptically. A serial decimal dilution was made from this suspension up to $1:10^{-10}$. 1 mL of each dilution was used to inoculate plates in triplicate containing specific growth media for different microorganism. The total colony forming units (cfu) of bacteria, fungi and actinomycetes were recorded on Gauze No. 1 agar (for actinomycetes, Ye et al., 1983), Martin's rose bengal streptomycin agar agar (for fungi, Martin, 1950) and Jensen's agar (for bacteria, Jensen, 1951) media, respectively. There were nine plates for each sample, and the plates were incubated at 28°C for 2–4 days for bacteria, 5–8 days for fungi, and 11–14 days for actinomycetes; after, microbial population was calculated and expressed as 10^n cfu/g air dried soil, where 10^n was dilution factor.

Determination of enzymatic activity for *Morchella* grown soil

Soil samples were collected on the same time as the enumeration of major soil microbial population. The samples for the determination of enzyme activities were sieved (1 mm) after being air-dried at room temperature for 7 days and stored at 4°C prior to analysis. The studied soil enzymes were invertase, urease, polyphenol oxidases and proteases. Invertase activity in soil was assayed by measuring the release of reducing sugar (Papa et al., 2010). Soil urease activity was determined by the method of Tabatabai and Bremner (1972) and protease activity by the method of Ladd and Butler (1972) using gelatin as the substrate. Polyphenol oxidase activity was measured by absorbance at 420 nm using the method described by Kar and Mishra (1976) and pyrogallol used as the substrate.

Statistical analysis

All the experimental results were means±SD and statistical significances of differences among treatments were determined by using the Statistical Package for the Social Sciences (SPSS) (version 13), and followed by comparisons at significance level of 0.05 ($P<0.05$).

RESULTS AND DISCUSSION

Morchella strain identification and its sclerotia observation by SEM

The edible mushroom *Morchella esculenta* Pers. forms sclerotia, whose importance in its life cycle was demonstrated by Ower et al. (1986). Amir at al. (1995) compared nine inhibitors affecting translocation and sclerotia formation in *M. esculenta* and they found that the use of nikkomycin inhibited sclerotia formation, without affecting translocation to the sclerotia. Therefore, it is very important to qualify the formation of sclerotia for the experimental strain. Results of scanning electron microscopy (SEM) had shown that *Morchella* sclerotia were formed by tube-shape mycelium and looked like granules (Figure 1). From the photos shown in Figure 1, we found that the sclerotia of *M. conica were* about 15±3.24 µm in diameter, and its mycelium about 10.25±2.13 µm in diameter. Some mycelia were observed to create plasma bridges and such results were reported by others (Guler and Ozkaya, 2008; Arkan 1992). Volk and Leonard (1990) reported that one area of neglect is the cytology of various development stages of the *Morchella* life cycle: the vegetative hyphae, sclerotia and fruiting bodies. Guler and Ozkaya, (2008)

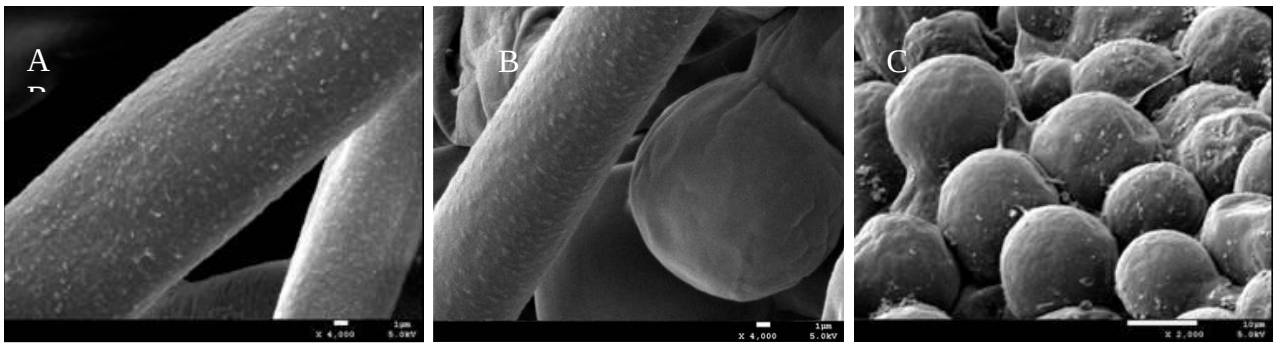

Figure 1. Mycelium and sclerotia observation by Scanning emission microscope for *Morchella conica.* Figure 1A, mycelium of *M. conica* observed by SEM with the mycelium shaped as a kind of tube coated with small particles. Barrel-shaped cell in sclerotia. Bar= 1 µm. Figure 1B, sclerotia of *M. conica were* about 15±3.24 µm in diameter, and its mycelium about 10.25±2.13 µm in diameter. Bar= 1 µm. Figure 1C, round, closely packed basal sclerotia.

investigated the effects of various carbon sources for *M. conica* sclerotium formations under in vitro conditions and found sclerotia cells were generally very thick and spherical. It was thought that the angle of hypha growth was something collected with sclerotia in culture medium, but this needs further confirmation.

Former species-level molecular systematic markers employed within this genus were limited to isozymes (Gessner et al., 1987; Royse and May, 1990; Wipf et al., 1996), restriction fragment polymorphisms of the LSU rDNA (Bunyard et al., 1994, 1995), ITS region of the nuclear rDNA (Buscot et al., 1996), and phylogenetic analyses of the ITS rDNA sequence data (Wipf et al., 1999; Kellner et al., 2005). Using multilocus DNA sequence data and phylogenetic species recognition (Dettman et al., 2003; Taylor et al., 2000), researcher investigated DNA sequences from marker loci，including ITS rDNA, and partial sequences of LSU rDNA, RPB1, RPB2, and EF-1 alpha to study morels species limits and to assess evolutionary relationships (O'Donnell et al., 2011; Tas-kın et al., 2010, 2012; Du et al., 2012a). Du's research also found that ITS sequence data has limited utility in differentiating species within the species-rich Elata sub-clade in the identified three lineages within *Morchella* (Du et al. 2012b) and most *Morchella* species appear to exhibit continental endemism and provincialism (O'Donnell et al., 2011). Because two-thirds of the *Morchella* ITS rDNA sequences identified to species in GenBank are misidentified and the ITS rDNA partition still was the most length variable (Du et al., 2012a), the *Morchella* strain in the present study was identified by *Morchella* MLST database in preference to NCBI GenBank numbers. Therefore, the studied strain was authenticated by analyzing its partial ITS rDNA sequences (750bp, Wipf et al., 1996) and the homologies analyses with *Morchella* MLST database with GenBank as a reference. Results are shown in (Figure 2).

The ITS sequences of experimental strain matched *M. conica*, with best matches characterized by minimum 98.5 % homologies to DQ257343(*M. conica*, China) and maximum 100% homologies to AJ544198 (*M. conica*, North Israel; Kellner et al., 2007) with NCBI. 13 homologous sequences within *Etala* clade were analysed, it is apparent that all of the black species of *Morchella* grouped closely. It shares 98.71 homologies to that of JF908365 (*M. conica,* Italy*)* within BioloMICS software (Robert et al., 2011), significantly, which shares 100% homologies to that of AJ544198 (*M. conica*, North Israel), shares over 99% homologies to EF080999 (99.9%; *M. conica*, China), AJ544194 (99.9%; *M. conica*, Germany), GQ265899 (99.7%; *Morchella costata,* China), AM269501 (99.7%; *M. conica*, Poland). These results obviously agree that variation may occur between geographically isolated populations of the same species (Bunyard et al., 1994).

ITS rDNA sequences give some information to analyses the commercial cultivation strain. Conducting these sequences, either through the species identification or possibly establish species DNA barcoding, we can better contribute to more suitable breeding in future. From above results, we found a very interesting phenomenon, that the present study strain of *M. conica* are just the same as another strain sampled from North Israel when their ITS rDNA sequences were compared, which suggested disjunct distribution during their evolutionary relationships.

Enumeration of major microbial populations from grown soil for *M. conica*

Up to now, we know little about the changes of the microorganisms from the cultured morel soils, especially treated with microelements such as ferric, zinc, among others. Microorganisms require iron for growth. Under iron-limited conditions, low molecular weight compounds called siderophores are synthesised by microbes (Varma and Chincholkar, 2007). Siderophores chelate ferric ions

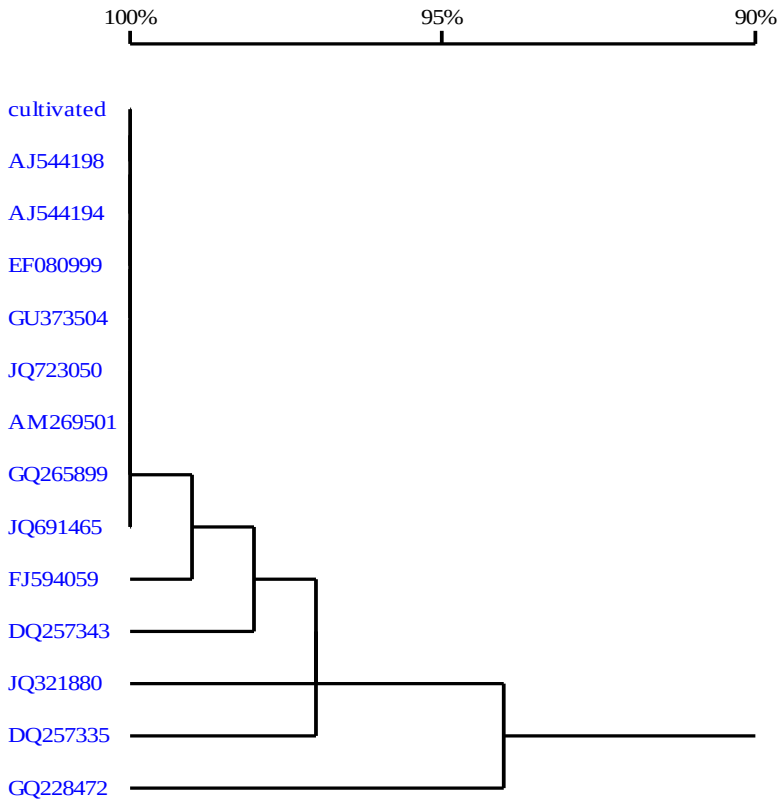

Figure 2. Cultivated strain of *Morchella conica* compared with other reported *Morchella spp.* for ITS1 sequences. AJ544198 (*Morchella conica*, Israel), AJ544194 (*Morchella conica*, Germany), EF080999 (*Moechalla conica*, China), GU373504 (*Morchella esculenta*, Finland), JQ723050 (*Morchella importuna*, China), AM269501 (*Morchella conica*, Poland), GQ265899 (*Morchella costata*, China), JQ691465 (*Morchella vulgaris*, Germany), FJ594059 (*Morchella conica*, Poland), DQ257343 (*Morchella conica*, China), JQ321880 (*Morchella populiphila*, China,), DQ257335 (*Morchella angusticeps*, China), GQ228472 (*Morchella elata*, India).

and transport it to the cell via receptor-mediated way. As iron is an indispensable growth factor, microbes producing siderophores can capture it from the others. Iron-deprivation causes decreasing metabolic activity and stop cell growth. Pseudomonas species synthesise numerous iron-chelators, e.g. pyoverdin and pyochelin (Cox et al., 1981; Cox and Adams, 1985). These compounds play an important role in restricting the growth of fungi (Matthijs et al., 2007). In this study, the numbers of culturable microbial population in soil at different developing stages were measured and results (Table 1) showed that experimental groups were higher than the control in number of bacteria, fungi and actinomycetes ($P<0.05$).

In detail, groups treated with ferric citrate and Fe·EDDHA during spore-formation stage show no significant differences as compared to the control group in bacteria number, but the number of fungi significantly increased and actinomycetes' number were significantly decreased. During primordium differentiation stage, experimental groups except the groups treated with $Fe_2(SO4)_3 \cdot 9H_2O$, were all significantly increased in the bacteria, fungi and actino mycetes' numbers, but the group treated with ammonium ferric sulfate ($NH_4Fe(SO_4)_2.12H_2O$) during spore formation were higher than others (included the control group) in the microorganism number, and such tendency was found in soil microflora number during the primordium differentiation stage. It is interesting that numbers of soil microorganism showed a tendency of increasing from spore formation stage to primordium differentiation stage and reached the highest, and then decreasing the microflora numbers when the *M. conica* developed into the fruit-body growth stage, but numbers of fungi were relatively changed lower than others. Generally, the bacterial and fungi population increased significantly from spore-formation stage to the primordia formation stage and increased up, then decreased at fruit-body maturing stage while the actinomycetes did not show the same

order. Group with ammonium ferric sulfate [$NH_4Fe(SO_4)_2.12H_2O$] often had the highest number compared to others at different stages. So, these results suggested that numbers of microorganisms of the grown morel soils differ in different ferric fertilizers. From these results, we also think that it is necessary to use the denaturing gradient gel electrophoresis (DGGE) technique with environmental samples to analyze the genetic diversity of microflora populations, especially in DGGE study of fungal communities (Vainio and Hantula, 2000; Anderson et al., 2003).

Determination of enzymatic activity for *Morchella* grown soil

Soils were sampled from three stages which morel grows. At all sites there was a significant treatment effect for each enzyme tested ($P<0.05$). The data suggested that four soil enzyme activities increased with the morel growing and reaches a maximum at primordia formation stage. *M. conica* grown soil with the ferric fertilizers' disposal at different stages had different effects on four soil enzyme (urease, polyphenol oxidase, invertase and protease) (Table 2). From the results, we found that four soil enzymatic activities increased with the morel growing and reaches a maximum at primordium differentiation stage. Among the four soil enzymes, polyphenol oxidase, invertase and protease showed a higher activity than urease. Group with ammonium ferric sulfate showed higher urease activity than those of other groups, probably due to the organic material or the greater nitrogen inputs and sub-sequent stimulation of microbial activity. Experimental groups showed higher activities in soil enzymes when compared to the control on a large scale. The data suggests that four soil enzyme activities increased with the morel growing and reaches a maximum at primordia formation stage. Among the four soil enzyme, polyphenol oxidase, invertase and protease showed a higher activity than urease.

During spore-formation stage, soil urease activities showed a tendency with $NH_4Fe(SO_4)_2.12H_2O$>$FeSO_4{\cdot}7H_2O$>$Fe_2(SO_4)_3{\cdot}9H_2O$>Fe·EDDHA and Ferric citrate> Nutrients>ck, and significant difference were shown between experimental groups except Nutrients and control group ($p<0.05$), especially the group treated with ammonium ferric sulfate[$NH_4Fe(SO_4)_2.12H_2O$] had been increased 8 times than control ($p<0.01$). Soil polyphenol oxidase activities of the experimental groups, except the group treated with Fe·EDDHA, showed significant differences when compared with that of the control. Invertase activities of the experimental groups were changed a little but not significantly different when compared with the control, while that of groups treated with $FeSO_4{\cdot}7H_2O$ and Nutrients were significantly decreased. Soil protease activities of the groups treated with Nutrients, $NH_4Fe(SO_4)_2.12H_2O$ and Fe·EDDHA were significantly increased when compared with that of the control. These results concludes that *M. conica* grown soil's enzymatic activities were effected by different ferric fertilizers, however, ammonium ferric sulfate ($NH_4Fe(SO_4)_2.12H_2O$) and Fe·EDDHA relatively showed higher soil enzymatic activities than others.

Urease catalyzes the hydrolysis of urea into ammonia or ammonium ion depending on soil pH, and carbon dioxide. Urease is the most prominent involved in soil N cycling in the four enzymes (Tabatabai and Bremner, 1972; Cookson, 1999). Among the different enzymes in soil, dehydrogenase, urease and phosphatases are important in the transformation of plant nutrients (Gao et al., 2010). We know very well that morels, like other edible macrofungi, their coarse proteins, nitrogen uptake levels were influenced by soil urease and protease. Such results perhaps indicated increased soil urease activities was necessary in order to promote the growth of morel hyphae, accumulate the yield of morel fruiting bodies and lower yield of commercial cultivation of *M. conica* which is as a result of the lower level of nitrogen application.

Results of the commercial planting for *M. conica*

In this study, the epigeal fructifications (ascocarps) of *M. conica* are usually appeared in late February or in early March when soil temperature warms to above 10°C and this is in accordance with previous reports (Kanwal and Reddy, 2012; Masaphy, 2010; Goldway et al., 2000; Pilz et al., 2007; Ower et al., 1986). A number of studies point out that obtaining sclerotia are a necessary part of the process for domestication of the genus and commercial production (Buscot, 1994). Also, we found obvious stage of sclerotial formation in our cultivation period of *M. conica*.

In this study, each group fruiting season length (d) was also used to characterize morel fruit body production. Most groups seemed to harvest in 18-22 days, which showed fruiting season length was not significantly correlated with any fertilizer, still positively affected by the air temperature and soil temperature (Mihaila et al., 2007). The yield results are shown in Figures 3 and 4.

In our experiment, most experimental groups, except the group with ferric sulfate [$Fe_2(SO4)_3.9H_2O$], increased the yields of *M. conica*; especially the groups with Fe·EDDHA and ammonium ferric sulfate [$NH_4Fe(SO_4)_2.12H_2O$] resulted in higher yields of fruit-bodies than other experimental groups and higher than that of control group, 1.77 and 1.88 times of the control yields, respectively. We found that during primordial formation of morel, groups treated with ferric fertilizers appeared faster than the control, approximately three or four days earlier, with a highest soil microbial biomass and enzymatic activities. Maybe *M. conica* is responding to the different strategies

Table 1. Numbers of major soil microbial population groups from the *Morchella* grown soil.

Group	Bacteria (10^6 cells/g dry soil)			Fungi (10^4cells/g dry soil)			Actinomycetes (10^4 cells/g dry soil)		
	Sclerotia formation	Primordium differentiation	Fruit-body mature period	Sclerotia appearing	Primordium differentiation	Fruit-body mature period	Sclerotia appearing	Primordium differentiation	Fruit-body mature period
Citrate ferric	1.32±0.07^{a}	5.90±1.13^{a}	1.43±0.06^{a}	3.59±0.26^{a}	4.40±0.48^{a}	5.32±0.10^{a}	4.38±0.29^{a}	3.48±0.28^{a}	3.57±0.24^{a}
Fe·EDDHA	0.92±0.13^{a}	4.79±0.59^{b}	2.25±0.10^{b}	2.94±0.57^{ab}	5.34±0.15^{b}	1.14±0.06^{b}	1.53±0.85^{b}	2.78±0.12^{b}	4.60±0.07^{b}
$Fe_2(SO4)_3.9H_2O$	2.63±0.34^{b}	3.26±0.21^{c}	3.94±0.06^{c}	2.50±0.73^{b}	4.56±0.21^{a}	1.64±0.13^{c}	3.75±0.06^{c}	6.30±0.23^{c}	3.76±0.10^{a}
$Feso_4.7H_2O$	3.27±1.36^{c}	6.37±0.11^{a}	3.18±0.11^{d}	2.25±0.28^{b}	6.43±0.06^{c}	3.71±0.17^{d}	5.72±0.29^{d}	6.44±0.10^{c}	6.62±0.18^{c}
$NH_4Fe(SO_4)_2.12H_2O$	3.21±0.39^{c}	8.71±0.17^{d}	1.66±0.17^{a}	5.87±0.51^{c}	8.45±0.29^{d}	5.65±0.12^{a}	5.47±0.24^{d}	6.78±0.14^{d}	5.42±0.11^{d}
Nutrients	2.07±0.29^{b}	5.48±0.52^{ab}	1.48±0.10^{a}	6.54±0.12^{c}	6.68±0.13^{c}	1.36±0.35^{bc}	3.56±0.21^{c}	5.45±0.15^{e}	4.67±0.22^{b}
ck (pure water)	1.75±0.140^{a}	3.53±0.06^{c}	3.80±0.26^{c}	1.45±0.50^{d}	2.43±0.06^{e}	3.55±0.16^{e}	2.36±0.16^{e}	3.43±0.19^{a}	3.60±0.12^{a}

Mean values as M±SD. Within three mean values of each character, there is no significant differences between those contain same letters, and different letters in the same column denote significant differences among treatments according to ANOVA and LSD's multiple comparisons ($p<0.05$).

Table 2. Soil enzymatic activities of the *Morchella* grown soil which treated by different ferric fertilizers.

Group	Sclerotia formation				Primordium differentiation stage				Fruit-body mature period			
	Urease	Polyphenol oxidase	Invertase	Protease	Urease	Polyphenol oxidase	Invertase	Protease	Urease	Polyphenol oxidase	Invertase	Protease
Citrate ferric	0.84±0.13a	3.55±0.55a	2.48±0.22a	3.34±0.10a	1.43±0.06a	6.52±0.59a	3.35±0.15a	5.44±0.10a	1.54±0.21a	6.35±0.02a	3.33±0.10a	4.53±0.15b
EDDHA.Fe	0.84±0.03a	2.36±0.36b	2.77±0.20c	4.53±0.03b	1.25±0.10b	4.20±0.62b	5.55±0.10b	6.47±0.61b	2.46±0.64b	3.55±0.15c	4.76±0.25b	3.33±0.01a
$Fe_2(SO4)_3.9H_2O$	1.09±0.36c	4.32±0.10c	2.16±0.53e	3.57±0.32d	1.55±0.15a	6.47±0.75a	6.63±0.06c	5.44±0.06a	1.55±0.15d	5.42±0.15d	3.35±0.26a	3.49±0.50d
$Feso_4.7H_2O$	1.23±0.20d	6.54±0.10d	1.36±0.61f	2.72±0.15e	3.14±0.20c	7.46±0.71c	3.34±0.78a	6.54±0.06d	3.41±0.06b	5.38±0.21d	2.76±0.44d	5.66±0.20e
$NH_4Fe(SO_4)_2.12H_2O$	4.28±0.04e	5.35±0.55e	2.92±0.01bc	4.22±0.10f	7.56±0.06d	7.33±0.10c	3.02±0.19d	2.56±0.10e	5.75±0.81c	6.36±0.46a	2.62±0.05e	3.32±0.06a
Nutrients	0.76±0.36b	3.51±0.35a	1.07±0.10d	6.36±0.49c	1.16±0.32b	4.19±0.21b	5.55±0.13b	7.48±0.53c	1.55±0.46a	3.44±0.10b	4.44±0.17c	2.44±0.15c
Ck (pure water)	0.53±0.11a	2.49±0.47b	2.74±0.08b	2.55±0.15g	3.20±0.26c	3.36±0.06d	2.70±0.08e	3.12±0.12f	2.45±0.25b	3.43±0.10b	2.67±0.21f	3.34±0.53a

Urease, [NH_3-N/ (mg $g^{-1}24^{-1}$)]; polyphenol oxidase, [gallic acid/(mg $g^{-1}24^{-1}$)]; invertase, [glucose/(mg $g^{-1}24^{-1}$)]; protease [amino acid/(mg $g^{-1}24^{-1}$)].

for growth and sclerotia formation. The result of better morel growth might be with the increasing soil microbial population due to fertilizer application, higher release of root exudates and larger amounts of root residue left in the soil, which positively influence microbial processes and development. This indicates that different ferric fertilizers could influence the primordial formation of morel and affect the yield of fruiting bodies by its soil microbial population and enzymatic activities. In nature, morel fruiting body formation is associated with a broad range of environmental stress conditions, some of which have yet to be defined (Masaphy, 2010) and up to now, there is no report about morel fruiting bodyformation and yield is associated with microelements, such as ferric, zinc, copper, molybdenum, among others. However, many literatures reported that microelements influenced the sclerotial formation and morels are rich in trace elements in their fruit-body (Kanwal and Reddy, 2012; Kalač, 2010; Amir et al., 1995). Fe·EDDHA is usually used to correct iron chlorosis in Strategy I plant, because of its great stability and solubility at high pHs (Rojas et al., 2008)

Figure 3. Artificial cultivation of morels. Figure 3A, milky sclerotia produced by *M. conica*, after incubated to puffed wheat soil media; Figure 3B, seed-planting. *Morchella* growing situation after hybridization, the plastic packages were equipped with the sterilized mixture including wheat grain, sawdust, chaff and vermiculite. They were used until the mycelium grown well; Figure 3C, morel growing period, is relative to certain temperature and humidity. Fruiting season length is not significantly correlated with any fertilizer; Figure 3D, fruit bodies in harvest time. The mature fruiting body reached 8–16 cm in length. The phenotypic features-fruiting body shape, color and size, are vary with, and affected by environmental conditions and developmental dynamics.

2008). In our study, it shows a higher effect on morel fruiting body than other ferric fertilizers did; the possible reasons may come from its great solubility. Nevertheless, the functional mechanisms of effects on different ferric fertilizers or other nutrition elements need to be further researched. In addtion, we think the same microelement or mineral element may influence the nutrient uptake for edible fungi by different approaches, it is difficult to give a sole conclusion whether a nutrient element promotes or hinders the experimental fungi to growth or even influence its yield at last. The best bet to optimize the nutrition plan for planting edible fungi especially *Morchella* spp. is to select a lot of nutrients (including trace elements) and to analyses their actual efficacy.

Conclusion

This paper reports studies on the effect of ferric fertilization on microbial biomass, enzymatic activities and morel yield. Sequencing of ITS showed that the species is closely related to *M. conica.* The results showed that among the six ferric fertilizers treatments, Fe·EDDHA, the ammonium ferric sulfate [$NH_4Fe(SO_4)_2.12H_2O$] results in the highest amount of microbial biomass , enzymatic activities and morel yields, while the treatments of CK gave the opposite results.

China is rich in nearly 30 morels species with eleven newly discover nine species within the Esculenta clade (yellow morels) and two novel species within the Elata clade (black morels), Over the 30 species, 20 appear to be endemic. The annual export of dried morels has been increased fivefold over the past 5 years to 900,000 kg, averaging $160 US dollars per kilogram (Du et al., 2012a). With their ever-increasing popularity, it is possible to purchase dried morels in local supermarkets in numerous countries throughout the year. Up to the present day, we have well known *M. conica* of its stable and effective planting condition. The study indicates ferric fertilizers making a big difference on the primordial formation of morel, further attempts regarding optimizing nutrition plan need to be carried out.

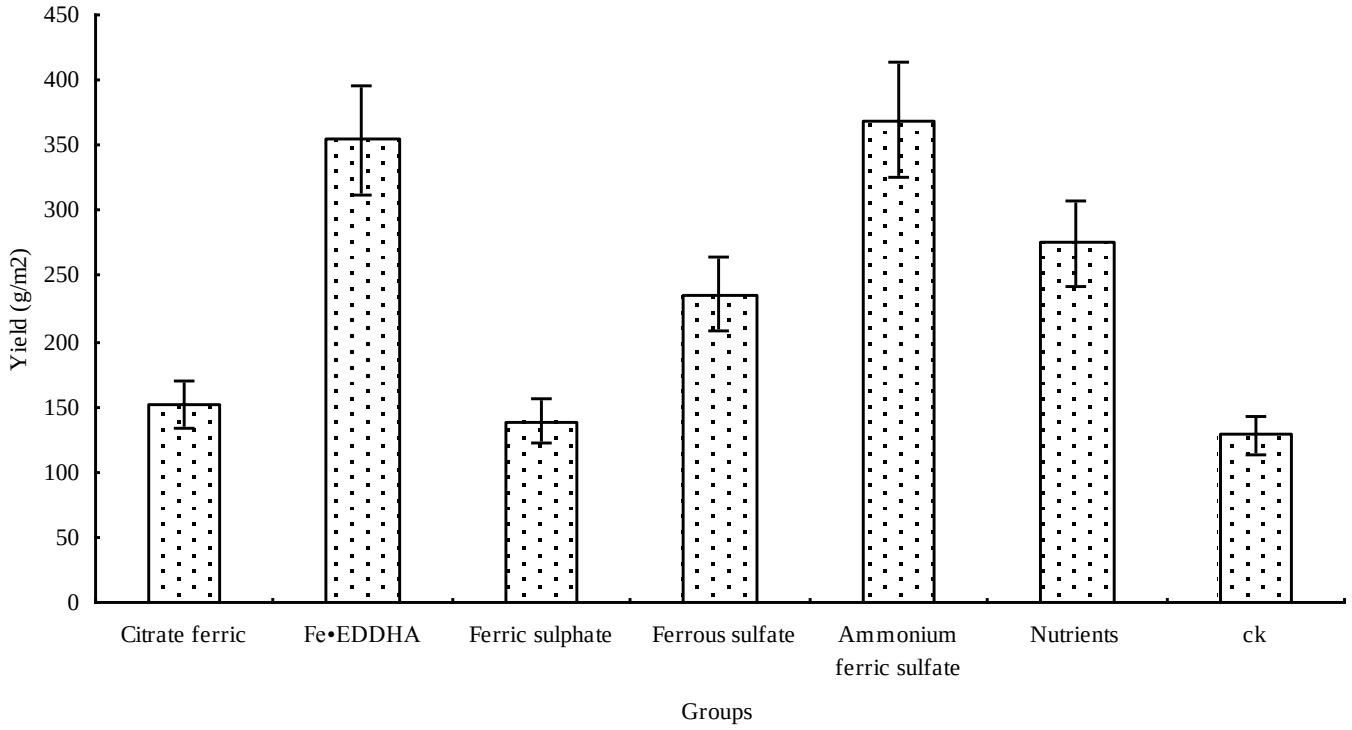

Figure 4. Yields of *Morchella conica* treated by different ferric fertilizer.

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Full Length Research Paper

Analysis of an outbreak of *Klebsiella pneumoniae* by the DiversiLab system and pulsed-field gel electrophoresis

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The aim of this study was to analyse the reason that five patients associated with bloodstream infections by *K. pneumoniae* in a surgical ward using Pulse Field Gel Electrophoresis (PFGE) and the repetitive-sequence-based PCR (rep-PCR) employing the DiversiLab system. In July 2010, eight isolates were collected from the same surgical ward of a hospital, and the specimens from sputum, blood and abdominal drainage fluid, respectively. The PFGE patterns after XbaI digestion and rep-PCR profiles produced by the DiversiLab system were determined for eight isolates. The Rep-PCR profiles produced by using the DiversiLab system showed that the eight isolates can be divided into two groups; the K8-02 as a group and the other seven strains is the other group. The eight strains of *K. pneumoniae* can be divided into two groups (A and B), seven strains were type A which the subtype A1 is the main-type (K8-01, K8-03, K8-04, K8-06, K8-08), and one is type B (K8-02). The PFGE and rep-PCR interpretations were concordant for the eight strains of *K. pneumoniae*. This data suggest that the DiversiLab system may be a reasonable alternative to PFGE for investigation and control of nosocomial infection outbreaks caused by *K. pneumoniae*, since it is easy to use, rapid and does not require highly skilled operators (Mazzariol et al., 2012).

Key words: *Klebsiella pneumoniae*, outbreak, pulse field gel electrophoresis (PFGE), rep-PCR, DiversiLab system.

INTRODUCTION

Klebsiella pneumoniae strain is one of the most important gram-negative pathogens causing hospital-acquired infection and community-acquired infection (Chen et al., 2013). In recent years, the infection and drug resistance of *K. pneumoniae* strain have been significantly increased; especially the growing in number of ESBLs-producing strains has become a major cause of death in patients with pneumonia. Therefore, rapid and accurate pathogen traceable epidemiological method is extremely important for the blocking and control of hospital infection epidemic spread. Now the major genotyping method for *K. pneumoniae* strain is PFGE, which is recognized as the gold standard of bacteria typing method with the highest discriminatory power (Han et al., 2013). However, PFGE requires specialized equipment and is technically demanding, labor intensive, and relatively slow, as it may take 2

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Abbreviations: ESBLs, Extended spectrum beta-lactamase; **CLSI**, clinical and laboratory standards institute; **PFGE**, pulse field gel electrophoresis; **rep-PCR**, repetitive-sequence-based PCR; ***K. pneumoniae***, *Klebsiella pneumoniae*.

Table 1. The clinical data of 8 *K. pneumoniae* strains.

Strain	Ward code	Patient code	Specimens type	Collection time
K8-01	S1	W	Sputum	July 17, 2010
K8-02	S1	W	Abdominal cavity drainage liquid	July 17, 2010
K8-03	S1	W	Blood	July 29, 2010
K8-04	S1	W	Blood	July 18, 2010
K8-05	S1	L	Blood	July 20, 2010
K8-06	S1	H	Blood	July 21, 2010
K8-07	S1	Y	Blood	July 29, 2010
K8-08	S1	J	Catheter	July 29, 2010

to 5 days to obtain results, depending on the organism and the methods utilized (Ligozzi et al., 2010). DiversiLab system is a typing method based the principle of rep-PCR, with the use of standardized operating, the high-resolution microfluidic chip and fluorescence detection system and an unified data processing software, has good repeatability, and can be used in the laboratory long-term epidemiological typing methods. It has been applied in *Acinetobacter baumannii* (Fontana et al., 2008), *Staphylococcus aureus* (Ross et al., 2005), *Pseudomonas aeruginosa* (Doléans-Jordheim et al., 2009), *Mycobacterium* (Cangelosi et al., 2004), and other molecular epidemiological studies. At present, reports on the DiversiLab system are rarely seen in China. In this study, we use the DiversiLab system to analyse genotypes of 8 *K. pneumoniae* strains, and compare the consistency between the DiversiLab system and Pulse Field Gel Electrophoresis (PFGE).

MATERIALS AND METHODS

Bacterial

The eight *K. pneumoniae* strains were collected from one surgical ward of a hospital in July 2010. All the strains were identified by using VITEK-2 compact automatic system (biomérieux, Marcy l' Etoile, France). The clinical datas are shown in Table 1.

Antibiotic susceptibility tests

Antibiotic susceptibility test profiles for 11 antimicrobial agents including amoxicillin/clavulanic acid (AMC), aztreonam (ATM), ciprofloxacin (CIP), ceftriaxone (CRO), cefazolin (CFZ), ampicillin (AMP), meropenem (MEM), cefoperazone/sulbactam (CPZ/SU), cotrimoxazole (SXT), piperacillin/tazobactam (PIP/TA), gentamicin (GEN) were determined by using a broth microdilution method. Standards for antimicrobial susceptibility testing and interpretation were based on the CLSI 2011 guidelines (CLSI, 2011). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains for susceptibility testing.

Pulse field gel electrophoresis method

Genomic DNA was prepared as described previously (Gori et al., 1996; Pang et al., 2002), but with some modifications. *K. pneumoniae* isolates were grown overnight on Mueller-Hinton plate. Takings a few pure colonies with a sterile cotton swab and dissolving with 2.5 ml TE buffer, then making 4.0 McFarland units, and incubating at 37°C. 150 µl prepared specimens were removed, 20 µl proteinase K and 30 µl 10% SDS (final concentration is 1%) were added, and mixed to warm-up at 56°C. Taking 2% low melting point gel and the prepared bacteria to fill the mold with the proportion of 1:1, then it was solidified at room temperature for 30 min. Then adding the molding of the plastic block to 1 ml lysis buffer which contains 5 µl proteinase K, and incubating for 2 h at 54°C shaking-bed. Then washed 2 times with 50°C preheated sterile distilled water, 10 min for each time, and washed 3 times with 50°C preheated TE buffer, 10 min for each time. Remove sample plastic block and cut down about 2 mm gel, then immersed it in digestion system which contains 200 µl restriction endonuclease enzyme Xba I, then 37°C digested overnight. Attach the digested gel block to the end of the glue comb, then place the comb at horizontally inverted plastic mold. The melted 1% electrophoresis agarose which had balanced at 50°C water to the mold was added, and then solidi-ficated at room temperature for 30 min. Setting the electrophoresis conditions as follows: the electrophoresis buffer 0.5 × TBE, switching time of 4 ~ 30 s, the field strength is 6 V/cm, temperature at 14°C, the electric field angle of 120°, and the electrophoresis time of 18.5 h, then view the result with ethidium bromide staining under ultraviolet light observations, pulsed-field gel electrophoresis (Tenover et al.,1995).

To determine the relationship between the band patterns as the following principles by visual inspection: 1) the same strains: the restriction map with same size and number is considered to be the one strain (main stream type); 2) closely related types: the electrophoretic bands have 3 or less different bands with the main phenol-type isolates due to mutation, insertion, deletion or inversion are considered to be the subtype of the main type; 3) may related types: type with 4 to 6 bands different is considered to be of different type; 4) not relevant: has more than 7 different bands with the main type is considered no correlation in epidemiology.

Rep-PCR using DiversiLab system

Using MOBIO UltraClean (TM) microbial DNA extraction kit to extract bacterial DNA and adjusting the DNA concentration to 25 ~ 50 mg/L with visible spectrophotometer (NanoDrop ND-1000). Rep-PCR was performed by using the DiversiLab *K. pneumoniae* kit for DNA fingerprinting (bioMerieux, Marcyl'Etoile, France) according to the manufacturer's instructions. Preparing the 25 µl PCR amplification system: the 18 µl rep-PCRMM1, 2.5 µl GeneAmp10 × PCR buffer, 2.0 µl Primer Mix, 0.5 µl AmpliTaq DNA polymerase and 2 µl template. Thermal cycling parameters were as follows: initial denaturation of 94°C for 30 s, annealing at 50°C for 30 s, extension at 70°C for 90 s, as a cycle, a total of 35 cycles, and a final extension at 70°C for 3 min. Analysis of PCR products were implemented by using DiversiLab System (bioMerieux) in which the amplified fragment of various size and fluorescent intensities were separated

Table 2. MIC values (µg/ml) of 8 *K. pneumoniae* strains.

Strain	(AMC)	(ATM)	(CIP)	(CRO)	(CFZ)	(AMP)	(MEM)	(CPZ/SU)	(SXT)	(PIP/TA)	(GEN)
K8-01	8	<=1	1	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16
K8-02	>=32	<=1	>=4	<=1	8	>=32	<=4	<=16	<=20	>=128	<=1
K8-03	4	<=1	0.5	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16
K8-04	4	<=1	0.5	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16
K8-05	8	<=1	0.5	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16
K8-06	4	<=1	0.5	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16
K8-07	4	<=1	0.5	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16
K8-08	4	<=1	1	>=64	>=64	>=32	<=4	<=16	>=320	<=4	>=16

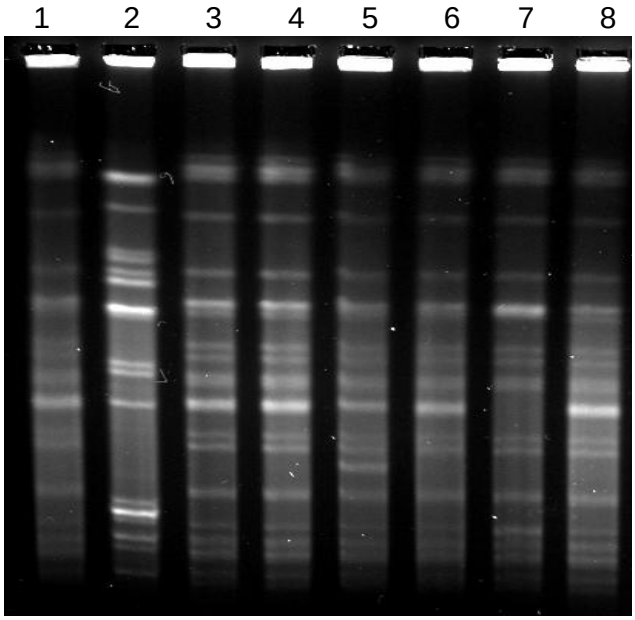

Figure 1. The PFGE typing results of eight *K. pneumoniae* strains. Note: 1 = K8-01, 2 = K8-02, 3 = K8-03, 4 = K8-04, 5 = K8-05, 6 = K8-06, 7 = K8-07, 8 = K8-08.

and detected using a microfluidics chip with the Aligent 2100 Bioanalyzer (Aligent Technologies, Santa Clara, CA, USA). Further analysis was performed with the web-based DiversiLab software version 3.3 with the Pearson correlation coefficient to determine distance matrices and the un-weighted-pair group method with arithmetic mean to create dendrograms.

The resulting DNA fingerprinting patterns were viewed as electropherograms, and the report included a dendrogram constructed from a similarity matrix and a virtual gel image of the fingerprint for each DNA sample. The criteria references of virtual gel image analysis are divided into three kinds: similar is greater than 97%, and no differences between bands; indistinguishable is greater than 95%, and has 1 to 2 different bands; different is less than 95%, and more than 2 different bands (Casolari et al., 2005).

RESULTS

Antibiotics susceptibility test

In 8 *K. pneumoniae* strains, the KB-02 was different with other isolates. The KB-02 was sensitive to SXT, GEN, MEM, CPZ/SU, CRO and ATM, and were resistant to AMC, CIP, CFZ, AMP and PIP/TA. The other 7 isolates were multiple drug-resistant *K. pneumonia*, which were sensitive to AMC, ATM, CIP, MEM, CPZ/SU and PIP/TA, and were resistant to CRO, CFZ, AMP, SXT and GEN. The results were in Table 2.

PFGE and rep-PCR using DiversiLab

The PFGE patterns after digestion with XbaI, and Rep-PCR using the DiversiLab system were performed for eight isolates. The eight isolates can be divided into two groups (A and B), seven strains were type A (K8-01, K8-03, K8-04, K8-05, K8-06, K8-07, K8-08), and one is type B (K8-02). Results are shown in Figure 1. The DiversiLab system showed the same results (Figures 2 and 3). The PFGE and the DiversiLab system interpretations were concordant for *K. pneumoniae* isolates. The DiversiLab system allowed a complete microbial typing analysis in approximately 4 h compared to 3 days for PFGE in our study.

DISCUSSION

There are many bacteria genotyping methods, such as DiversiLab system, MLST, PFGE and ERIC. Among them, PFGE is recognized as the gold standard of bacteria typing method. Witt (Te et al., 2009) applied the DiversiLab system, MLST and PFGE to study 93 MRSA strains, and the Simpson coefficient among three methods were 0.860, 0.877 and 0.905, respectively. Although, the resolution is different, the three kinds of methods are commonly used in genotyping of MRSA. Qu et al. (2010) used the DiversiLab system for genotyping of *A. baumannii*, which got good results. The DiversiLab system is fast and simple, besides its commercial kits, which is available for laboratory epidemiological typing methods. The eight *K. pneumoniae* strains were isolated from the same surgical ward of a hospital during July 2010, and the specimens from sputum, blood and abdominal drainage fluid, respectively. Antibiotics susceptibility test showed that among the eight strains, seven strains

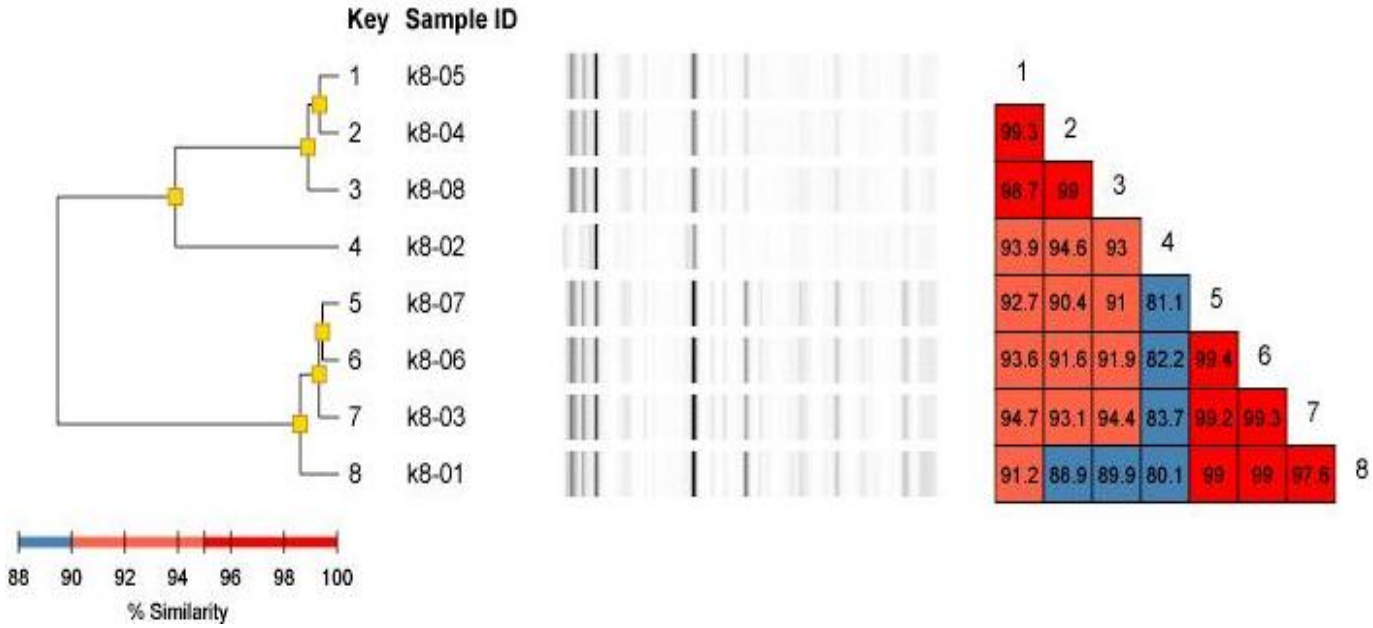

Figure 2. DiversiLab system typing results for *K. pneumoniae* strains; Note: The tree on the left side is the typing results of the DiversiLab system; figures in the right side of the matrix represent the similarity between the two strains, number on the right of the matrix represent strains, the Sample ID is the strain number.

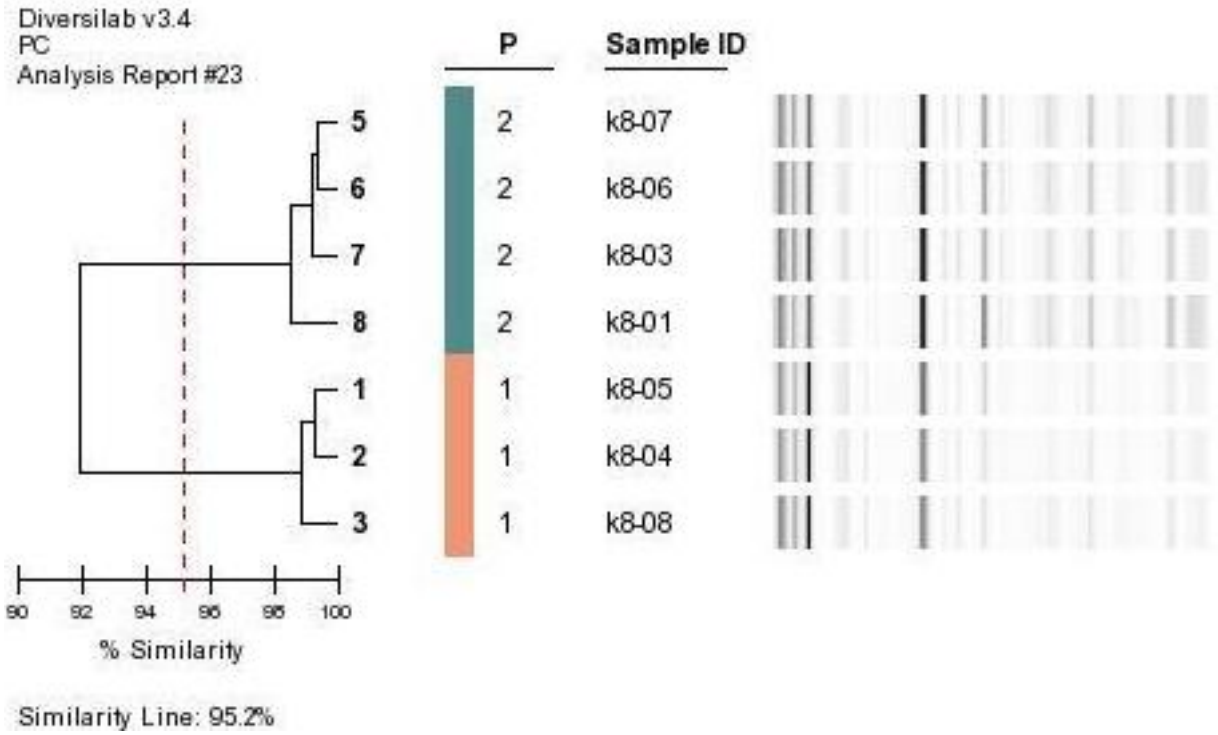

Figure 3. DiversiLab results of 7 *K. pneumoniae* strains except for K8-02 specimen.

have the same antimicrobial resistance pattern, except K8-2. The DiversiLab system and PFGE have proved these seven isolates belonging to the same type, so that the infection is likely an outbreak of hospital. The susceptibility pattern of the isolate K8-02 from abdominal cavity drainage fluid was different from three other strains

that came from blood and sputum samples, which were all from the patients W. And PFGE patterns and the DiversiLab system showed no correlation among these four *K. pneumoniae* strains.

According to the time of the five patients infected, the DiversiLab system analysis found that W was the first infection patient, and subsequently infected with the L, H, Y and J. Because these four *K. pneumoniae* strains were the same type, in addition to, the PFGE map also showed the same result. Therefore, the patient W was most likely the source of this outbreak of nosocomial infection, and spreaded to the patients L, H, Y and J through exogenous way. At present, the most common typing method for *K. pneumoniae* is PFGE, which is the gold standard of bacterial genotyping methods and homology analysis (Wise et al., 2009). But the PFGE results usually can be read with naked eye, and different operators will influence the result. Furthermore, the technology is time-consuming, because it normally takes 3~5 days to get the final results. On the other hand, DiversiLab system that based REP-PCR is a standardized and automated systems, and the result collection and analysis process has no subjective restrictions. It can analyse 12 specimens in 40 min and the total process only need 6~8 h. The DiversiLab system has high resolution, good repeatability and simple operation, is a real-time quantitative detection. But the system also has shortcomings.

The analysis is race operation that needs only the 1 µl each time, so that sometimes a bubble may need re-analysis of the amplified products and increases detection period and the cost. When using chip detection, even in the case of only several samples, the 12 sample holes and the standard hole must be added to the markers and gel fluorescent dye mixture, and result in reagent waste and cost. This study shows that the DiversiLab system is simple, fast, has high repeatability and high resolution, the results of quantitative processing, and various forms of output can be used as preferred genotyping method, especially on the short-term outbreak of a large number of specimens.

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Full Length Research Paper

Towards efficient crude oil degradation by *Pseudomonas* sp. strain-O2: Application of Plackett-Burman design for evaluation of cultivation conditions

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Towards an efficient crude oil bioremediation, optimization study of crude oil degradation by *Pseudomonas* sp. strain-O2 was done. Preliminary experiments of crude oil degradation revealed that peptone was the optimal nitrogen source recording 73.3% removal of crude oil, in the presence of 0.5 g/L of yeast extract. The increase of phosphate ratio to 2.5 led to 80.1% removal of crude oil. To evaluate the significance of different culture conditions that affect crude oil biodegradation, Plackett-Burman factorial design was applied. Eleven variables were simultaneously examined. Among those variables, crude oil concentration was the highest positively significant variable that encourage crude oil degradation in *Pseudomonas* sp. strain-O2 affecting the degradation process, other factors namely, Na-succinate, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4.7H_2O$ showed moderate effect. While, yeast extract, inoculum concentration, agitation, K_2HPO_4 and NaCl were the lowest significant variables. Fractional factorial design experiments indicated that the pre-optimized medium showed approximately 1.5-folds increase in crude oil degradation by *Pseudomonas* sp. strain-O2.

Key words: Biodegradation, crude oil, *Pseudomonas* sp., experimental design, optimization.

INTRODUCTION

As a result of industrialization, many harmful substances including the spill of crude oil and various oil residues have been discharged into terrestrial and aquatic environments (Margesin, 2000). Dangerous accumulation of recalcitrant compounds in soil, sediments and groundwater are considered to be a potential health hazards (Korda et al., 1997). The clean-up of petroleum hydrocarbon-contaminated sites remains a priority task for restoration of the natural environment (ASTM, 1995). Considerable efforts are being spent to design cheap and feasible strategies for clean-up of contaminated sites.

Recently, bioremediation has proved to be a useful tool in removing oil (Boopathy, 2000; Ruiz et al., 2006). It attempts to accelerate the natural degradation rates by overcoming factors that limit microbial degradation (Atlas, 1991). Therefore, bioremediation technology causes the improvement of the natural capacity of microorganisms to degrade contaminants (Catallo and Portier, 1992; Atlas, 1991; Salanitro et al., 1997). Variety of bioremediation methods have been developed to support and increase the degradation activities of native microbial populations, allowing reduction in time and subsequent saving in costs. The two main approaches to bioremediation are the biostimulation and bioaugmentation (Korda et al., 1997).

On the other hand, hydrocarbon-degrading microorganisms are ubiquitous in most ecosystems where contaminants may serve as organic carbon sources (Atlas and Bartha, 1992; Margesin et al., 2000). Bacteria are the most active agents in petroleum biodegradation and there is evidence of their fundamental role as primary degraders

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of spilled oil (Komukai-Nakamura et al., 1996; Ijah, 1998; Rahman et al., 2002; Head et al., 2006; da Cruz et al., 2011; Oliveira et al., 2012). Effect of various nutrients on the degradation of crude oil by different bacteria was investigated by several scientists (Gibbs, 1975; Dibble and Bartha, 1979, Wrenn et al., 1994; Oh et al., 2001; Berwick, 2004; Xu et al., 2005). Several factors, both physico-chemical and biological, affect the rate of microbial degradation of hydrocarbons in soil. Recently, growing interest in the use of several Pseudomonades during degradation of crude oil have been reported (Bosch et al., 1999; Evans et al., 2004; Wongsa et al., 2004; Meng et al., 2005; Emtiazi et al, 2005; Toledo et al., 2006; Song et al., 2006; Ueno et al., 2006; Das and Mukherjee, 2007; Mittal and Singh, 2009). However, application of statistical experimental design for optimization of crude oil degradation with *Pseudomonas* sp. was rarely investigated. Recently, medium optimization for a noval crude-oil degrading lipase from *Pseudomonas aeruginosa* SL-72 using statistical approaches for bioremediation of crude-oil was reported (Nain et al., 2012).

On the other hand, experimental design techniques present a more balanced alternative to one-variabler-at-a-time approach (OVAT) in which single factor is varied, while others are kept fixed. However, Plackett and Burman design comprise one type of two-level screening and can be constructed on the basis of fractional replication of a full factorial design (Plackett and Burman, 1947). This design is appropriate to face the large number of cultivation conditions under investigation and allow obtaining an unbiased estimates of linear effects of all factors with maximum accuracy for a given number of observations (Akhnazarova and Kafarov, 1982).

The main aim of this work was to investigate the possible improvement of crude oil degradation by *Pseudomonas* sp. strain-O2 and to evaluate the influence of different cultivation condition on efficiency of crude oil degradation. Preliminary controlled experiments were conducted to address the most effective nitrogen source and its optimal concentration, yeast extract, $MgSO_4.7H_2O$ and the level of phosphate salts that might affect crude oil degradation. To determine the significance among other physical and nutritional requirement, fractional factor design namely; Plackett-Burman experimental design was applied and the significant variables were determined.

MATERIALS AND METHODS

Microorganism and cultivation medium

Bacterial strain used in this study, *Pseudomonas* sp. strain-O2 was isolated and identified as previously mentioned (Mostafa et al., 2012). The bacterium was grown on minimal salts medium (MSM), it was the modified medium of Ijah (1998) with the following composition, (g/L): yeast extract, 0.5; NaCl, 0.5; $(NH_4)_2S0_4$, 2; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 5; KH_2PO_4, 2 and trace elements (with the following composition, (g/L): $FeSO_4$, 5; H_3BO_4, 0.025; $CuSO_4.5H_2O$, 0.005; KI, 0.005; $CoSO_4$, 0.3; $MnSO_4.4H_2O$, 3; $ZnSO_4.7H_2O$, 5; $NaMoO_4$, 0.012, and distilled water up to 1 L), 0.1 mL.

Monitoring of crude oil biodegradation

Biodegradation of crude oil was investigated by cultivation of the bacterium in 250-mL Erlenmeyer flasks containing 100 mL sterile MSM amended with 0.92% (w/v) crude oil as sole carbon source. 1.5 mL of active inoculum, prepared from 24 h preculture of the bacterial strain grown on nutrient broth medium, was used for inoculation. All flasks were incubated at 30°C under shake conditions at 120 rpm. At the end of 4 days incubation period, flasks were analyzed for the residual crude oil as described below.

Extraction of crude oil

For extraction of the residual crude oil remaining at the end of cultivation period, each flask was acidified with 5 mL H_2SO_4 (1:1) pH 2; then extracted three times by 60 mL methylene chloride in a 250 mL separating funnel, the organic layer was drained through a funnel containing anhydrous sodium sulfate into a 50 mL boiling rounded flask; then moved to a rotary evaporator at about 40°C to reduce the volume of the extract to 1 mL. The residual oil was transferred to a pre-weighed 2 mL vial for gravimetric analysis, where the amount of crude oil remaining was determined and the percent of crude oil removal was calculated.

Influence of medium composition on crude oil degradation

A series of preliminary on-variable-at-a-time (OVAT) experiments were carried out to provide information for determination of settings of variables that might be used during experimental design. The effect of different N-sources was firstly investigated, nitrogen sources used (2 g/L) were; peptone, ammonium sulfate, ammonium chloride or sodium nitrate. Other tested conditions included; different levels of peptone (2 to 5 g/L), inoculum concentration (0.5 to 2% v/v), yeast extract (0 to 2.5 g/L), $MgSO_4.7H_2O$ (0.1 to 0.5 g/L) and phosphate salts K_2HPO_4 and KH_2PO_4 (1- to 5-folds) the basal concentration (2:0.8 g/L).

Plackett-Burman design

Plackett-Burman experimental design most commonly used for screening purpose was applied to evaluate the significance of various medium components as well as environmental factors affecting crude oil degradation by *Pseudomonas* sp strain-O2. The different factors were prepared in two levels: -1 for low level and +1 for high level, based on Plackett-Burman statistical matrix design, which is a fraction of a two-level factorial design and allows the investigation of n-1 variables in at least n-experiments (Plackett and Burman, 1947). Eleven independent variables (Table 1) were screened in 14 combinations according to the design shown in Table 2. All trials were performed in triplicate and the average of observation was considered as the final result. The main effect of each variable was calculated simply as the difference between the average of measurements made at the high setting (+1) and the average of measurements observed at low setting (-1) of that factor. Plackett-Burman experimental design is based on the first order model (equation 1):

$$Y = \beta_0 + \sum \beta_i x_i \qquad (1)$$

Where Y is the predicted response (% removal of crude oil), β_0, β_i are constant coefficients, and x_i is the coded independent variables estimates or factors.

Analysis of data

The data of crude oil degradation was statistically analyzed.

Table 1. Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting crude oil degradation by Pseudomonas sp. strain-O2.

Code	Variable	Value	
		-1	+1
X_1	Inoculum concentration	0.5	1.5
X_2	Ammonium sulfate (g/L)	0.5	2
X_3	Peptone (g/L)	2	5
X_4	Yeast Extract (g/L)	0	5
X_5	$MgSO_4.7H_2O$ (g/L)	0.2	0.5
X_6	K_2HPO_4 (g/L)	2	5
X_7	KH_2PO_4 (g/L)	0.8	2
X_8	NaCl (g/L)	5	2
X_9	Agitation (rpm)	60	120
X_{10}	Oil Concentration (g/L)	0.5	1.5
X_{11}	Na-succinate (g/L)	0.5	2

Essential Experimental Design free software (Steppan et al., 2000) was used for data analysis and determination of coefficients. Factors having highest t-value and confidence level over 95% were considered to be highly significant on crude oil degradation.

RESULTS

Influence of medium composition by OVAT

Nitrogen, phosphate and sulfate levels

To provide information on the variable levels that might be used in experimental design study, a series of preliminary OVAT experiments were carried out. At the end of each experiment, cells were separated and the residual crude oil was estimated. Results in Figure 1 indicated that peptone was the optimal nitrogen source recording 73.3% removal of crude oil. Inorganic nitrogen sources such as; ammonium sulfate and sodium nitrate showed positive significance on the growth and crude oil degradation and recorded 71.1 and 69.6% removal of crude oil, respectively. Also, 2 g/L was the optimal peptone concentration, any further increase in concentration led to reduction in crude oil degradation efficiency (Table 3). On the other hand, to determine the most suitable level of yeast extract, MSM was amended with different concentrations of yeast extract ranging from 0 to 2.5 g/L. Results in Table 3 indicated that 0.5 g/L was the optimal concentration that led to approximately 83% removal of crude oil. Approximately, 60.4 and 52.9% removal of crude oil concentration was recorded when yeast extract was increased to 1 or 2.5 g/L, respectively.

Furthermore, one of the crucial chemical constituents in the medium that affect crude oil degradation is the phosphate salt. Results in Table 4 indicated that the increase of phosphate ratio to 2.5 led to 80.1% removal of amount of crude oil. Further increase or decrease in phosphate salt ratio led to decrease in oil degradation efficiency and hence in percent removal of crude oil. Indeed, the optimal concentration of $MgSO_4.7H_2O$ was 0.5 g/L recording approximately 60% removal of the crude oil (data not shown).

It is known that the concentration of the bacterial inoculum plays an important role in determining the efficiency of crude oil degradation. Results revealed that 1.5% (v/v) was the optimal inoculum concentration that led to approximately 80% removal of crude oil (data not shown).

Evaluation of cultivation condition by FFD

Fractional factorial design (FFD) is a kind of experimental design that enables researchers to evaluate the most significant factors affecting the process with the least number of trials. Plackett-Burman design is a FFD, which succeeds in ranking factors from different categories to enable better understanding of the medium effects, eleven factors were studied through the application of Plackett-Burman design. Evaluation of process variables was carried out according to the experimental matrix presented in Table 2, where the residual crude oil estimated and the percent of crude oil removal was the measured response. Variation in crude oil degradation expressed as % of crude oil removal (73 - 96.6%) is shown in Table 2. Results collectively showed that the highest crude oil removal of 96.6 was obtained in the combination number 9, while the lowest crude oil removal was obtained in combination numbers 6. Statistical analysis of these data revealed that the value of determination coefficient R2, that measures the goodness of the model fitting, is >0.99. This indicates that less than 1% of the total variations are not explained by the model, which ensures the good adjustment of the model.

Moreover, the main effects of the examined variables on degradation of crude oil were calculated and illustrated graphically in Figure 2a.

On analysis of regression coefficients and *t*-value (Table 5), it was clear that mainly crude oil concentration, together with $MgSO_4.7H_2O$, $(NH_4)_2SO_4$, KH_2PO_4 and Na-succinate were found to be the most significant variables that encourage crude oil degradation in *Pseudomonas* sp. strain-O2. Whereas, bacterial inoculum, yeast extract concentration, agitation and NaCl were the lowest significant variables that discourage crude oil degradation.

The determination coefficient represents the quality of fitting the polynomial model, which can be represented as follows:

$$Y_{\%\ removal} = 11.70 - 2.88X_1 + 0.7X_2 + 0.07X_3 - 4.16X_4 + 1.71X_5 - 0.46X_6 + 0.85X_7 - 0.29X_8 - 0.54X_9 + 6.11X_{10} + 0.52X_{11}$$

One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature (either nutritional or

Table 2. Plackett-Burman experimental design for evaluation of factors affecting crude oil degradation by *Pseudomonas* sp. strain-O2.

Experiment	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Oil removal (%)
1	1	-1	-1	-1	1	1	1	-1	1	1	-1	80.7
2	1	1	1	-1	1	1	-1	1	-1	-1	-1	92.3
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	90.0
4	0	0	0	0	0	0	0	0	0	0	0	88.0
5	1	-1	1	1	-1	1	-1	-1	-1	1	1	91.5
6	-1	1	1	-1	1	-1	-1	-1	1	1	1	73.0
7	1	1	-1	1	1	-1	1	-1	-1	-1	1	96.4
8	1	1	-1	1	-1	-1	-1	1	1	1	-1	92.4
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	96.6
10	1	-1	1	-1	-1	-1	1	1	1	-1	1	94.7
11	-1	-1	1	1	1	-1	1	1	-1	1	-1	81.4
12	0	0	0	0	0	0	0	0	0	0	0	88.0
13	-1	1	-1	-1	-1	1	1	1	-1	1	1	75.0
14	-1	1	1	1	-1	1	1	-1	1	-1	1	96.3

Variables coded as follows: X_1, inoculum concentration; X_2, ammonium sulfate; X_3, peptone; X_4, yeast extract; X_5, , $MgSO_4.7H_2O$; X_6, K_2HPO_4; X_7, KH_2PO_4; X_8, NaCl; X_9, Agitation; X_{10}, Oil concentration; X_{11}, Na-succinate.

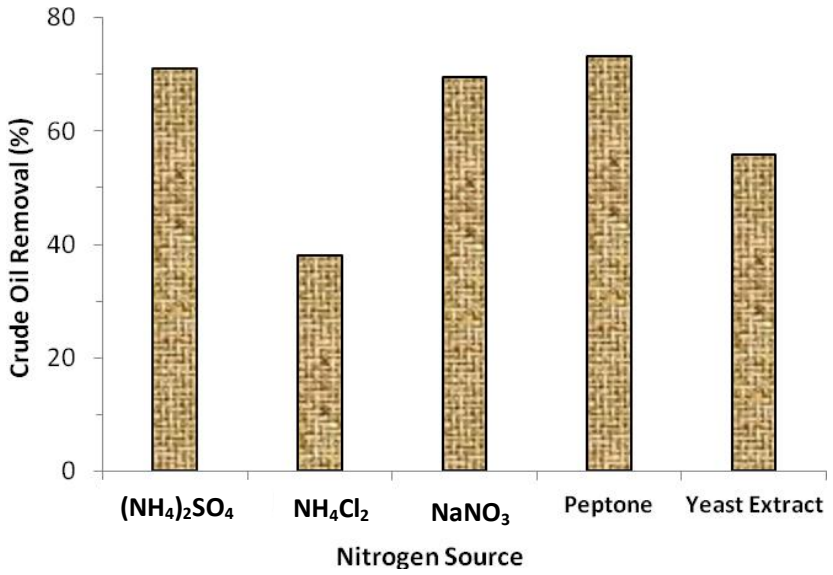

Figure 1. Effect of different nitrogen sources on degradation of crude oil by *Pseudomonas* sp. strain-O2.

physical factor) or sign (whether contributes positively or negatively). Interestingly, Figure 2b shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and is a convenient way to view the results of Plackett-Burman design (Strobel and Sullivan, 1999).

DISCUSSION

Given the environmental importance of bacteria, especially Pseudomonades, in degradation of crude oil and their application in bioremediation and namely, bioaugmentation and biostimulation, the influence of different growth parameters on degradation process was closely examined. In a series of controlled OVAT experiments, optimal concentrations of medium constituents were closely investigated. Preliminary investigations indicated that peptone is the optimal organic nitrogen source. Supplementation of the medium with yeast extract enhanced growth and % removal of crude oil from the culture medium reflecting its importance as nitrogen and vitamin source. The use of yeast extract as nitrogen as

Table 3. Effect of different peptone and yeast extract concentrations on crude oil degradation by Pseudomonas sp. strain-O2.

Peptone concentration (g/L)	Extracted crude oil (mg)	Crude oil removal (%)	Yeast extract concentration (g/L)	Extracted crude oil (mg)	Crude oil removal (%)
2	16.3	82.3	0	18.0	80.1
3	28.9	68.6	0.5	15.6	83.0
4	37.9	58.8	1.0	36.4	60.4
5	21.3	76.8	2.5	43.3	52.9

Table 4. Effect of different phosphate ratios on crude oil degradation by *Pseudomonas* sp. strain-O2.

K2HPO4 : KH2PO4 (g/L)	Fold	Extracted crude oil (mg)	Crude oil removal (%)
2 : 0.8	1	46.2	50.6
4 : 1.6	2	29.5	49.8
5 : 2	2.5	18.3	80.1
6 : 2.4	3	23.6	74.3
7 : 2.8	3.5	23.2	74.8
9 : 3.6	4.5	35.2	61.7
10 : 4	5	37.7	59.0

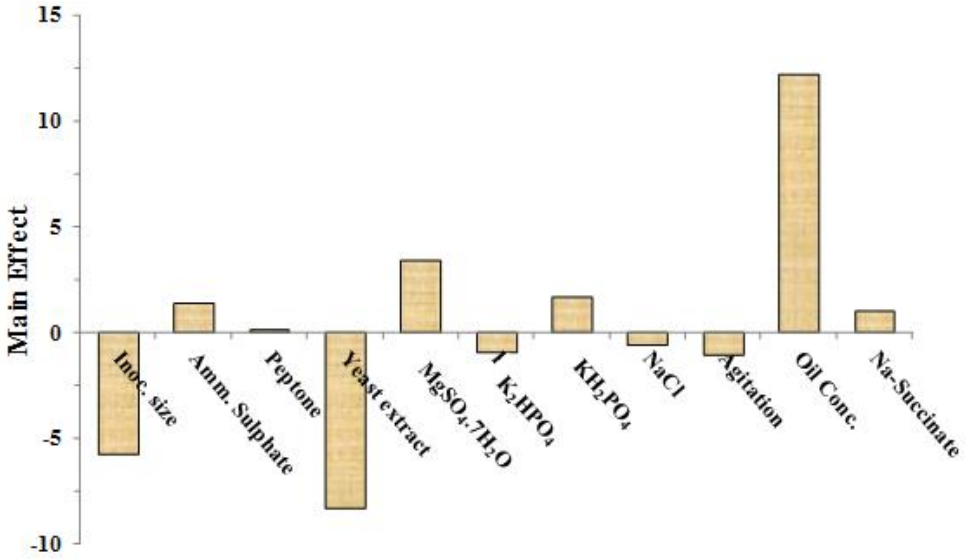

Figure 2a. Effect of different factors on crude oil degradation by *Pseudomonas* sp. strain-O2 as screened with Plackett-Burman design.

well as vitamin source during hydrocarbon and petroleum degradation by many organisms was reported (Lemos et al., 2002; Singh et al., 2005). Interestingly, increase in yeast extract concentration from 0.5 to 2.5 g/L led to approximately 20% reduction in crude oil removal. In concordance, results of Plackett-Burman experiments revealed that the increase in yeast extract concentration negatively affected crude oil degradation. On the other hand, one of the crucial chemical constituents in the medium that affect crude oil degradation is the phosphate salt, especially due to its suggested importance in indirect control of pH and providing the organism with the required phosphate ions necessary for energy and ATP production. Indeed, buffering effect of phosphate salts during degradation of crude oil was reported by Emtiazi et al., (2005). This finding supported by the results of fractional factorial design which indicated that phosphate ion (KH_2PO_4), contributed positively to crude oil biode-

Table 5. Statistical analysis of data obtained from Plackett-Burman design showing coefficient values, t- and P-values of each variable.

Variable	Statistical analyse			
	Coefficient	*t*-Stat	P-value	Coefficient level (%)
X_1	-2.88	9.98	0.009	98.4
X_2	0.70	-2.43	0.135	04.0
X_3	0.07	-0.24	0.828	99.8
X_4	-4.16	14.3	0.004	99.4
X_5	1.71	-5.91	0.027	93.5
X_6	-0.46	1.59	0.251	96.0
X_7	0.85	-2.95	0.098	89.0
X_8	-0.28	0.99	0.424	99.2
X_9	-0.53	2.00	0.183	95.6
X_{10}	6.11	-21.1	0.002	96.1
X_{11}	0.52	-1.54	0.261	48.4

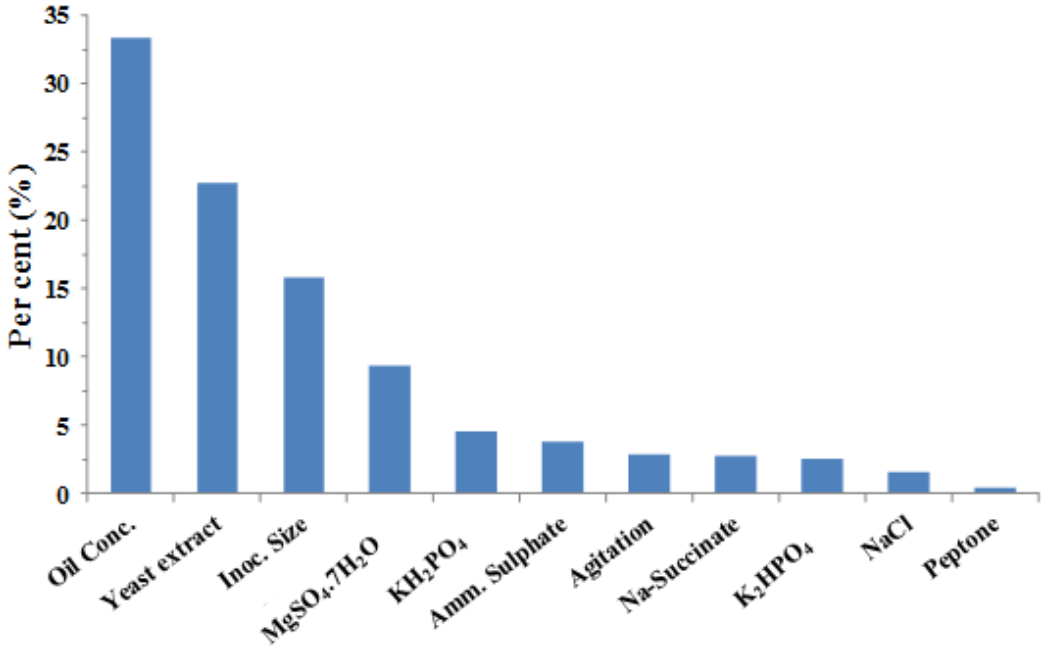

Figure 2b. Pareto chart rationalizing the effect of each variable on crude oil degradation by *Pseudomonas* sp. strain-O2.

gradation and showed high significant effect reflected by the p-value (0.098). Furthermore, supplementation of fermentation medium with inorganic nitrogen source such as $(NH_4)_2SO_4$ contributed positively to crude oil degradation, consequently can be used instead of other organic nitrogen sources. In accordance, Nain (2012) reported that ammonium and phosphate ions contributed positively to production of crude oil degrading lipase from *P. aeruginosa* SL-72 during application of Plackett-Burman experimental design. The use of inorganic nitrogen sources, such as ammonium chloride and potassium nitrate, was preferably used during crude oil degradation (Wrenn et al., 1994). Many scientists reported the importance of amendment of biodegradation medium with phosphate and inorganic nitrogen fertilizers (Westlake et al., 1978; Jobson et al., 1974; Piehler and Paerl, 1996; Emtiazi et al., 2005; Margesin et al., 2007). Furthermore, it was clear that the degradation efficiency was positively affected by the amount of crude oil due to the increase in the amount of C-source (p-value= 0.002). Similar finding was reported by Margesin et al. (2007) and Xu et al. (2005). Addition of Na-succinate as co-substrate to enhance crude oil degradation resulted in a slightly positive effect. Simple co-substrates were reported to

positively affect the rate of hydrocarbon degradation. Significant increase in degradation of crude oil as well as the saturated branched hydrocarbon (squalane) by addition of glycerol, rhamnolipid or Na-succinate was reported (Berekaa and Steinbuchel, 2000; Meng et al., 2005). Successful degradation of crude oil PAHs by co-metabolism was reported (Arun et al., 2011). Whereas, the other variables namely, agitation, NaCl and K_2HPO_4 were the lowest significant variables affecting crude oil degradation. Therefore, might be dropped in further optimization experiments.

The results of this study collectively revealed the possible optimization of crude oil degradation by *Pseudomonas* sp. strain-O2 through improvement of chemical and environmental parameters. Preliminary experiments gave an idea about the setting of some variables that might be used in experimental design. Plackett-Burman design showed that the most significant variables encourage crude oil degradation. Furthermore, the design succeeded to rank factors from different categories to enable better understanding of the medium effect. It is worthwhile to further optimize the significant variables determined in the present study to attain maximum crude oil degradation by applying other suitable statistical designs.

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Full Length Research Paper

Real time polymerase chain reaction for identification of *Candida* species isolated from blood stream infections in non-neutropenic cancer patients

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This work aimed at identifying *Candida* species isolated from blood stream infection in non-neutropenic cancer patients by conventional and molecular methods. Out of 200 blood samples from cancer patients with blood stream infection, 89 were positive for *Candida* which were identified using chromogenic medium (CHROMagar *Candida*), and real-time polymerase chain reaction (RT-PCR) assays targeting the fungal ITS2 region using a LightCycler instrument. The present study revealed that non-*albicans Candida* species caused most of the cases of candidaemia in the cancer patients. A total of 89 *Candida* isolates were obtained, *Candida albicans* represent 15/89 patients (16.85%) and non-*albicans Candida* species accounts for 74/89 patients (83.1%) of which *Candida tropicalis* represent 32.6% followed by *Candida parapsiliosis* 23.6%, and then *Candida rugosa* (21,4%) and the lowest percentage was in *Candida glabrata* (5.6%). The results of the study revealed that real time-PCR could detect 81 out of 89 cases of candidaemia with sensitivity of 91.01%, specificity of 95.6%, PPV of 94.18% and NPV of 92.30%. The high prevalence of non-*albicans* candidaemia should be considered prior to antimicrobical therapy in non-neutropenic cancer patients with candidaemia. Real-time PCR represents a rapid and accurate method for diagnosis of candidaemia.

Key words: Candidaemia, *Candida albicans*, real-time polymerase chain reaction (RT-PCR).

INTRODUCTION

Candida species are currently the fourth most common cause of bloodstream infections worldwide, and the third most common cause of bloodstream infections in patients with malignancy (Chung et al., 2005). Candidiasis is not only associated with mortality of about 30to 40% in cancer patients, but also extends the duration of hospital stay and increases the cost of medical care (Naglik et al., 2004). The clinical presentations of patients with blood stream infection caused by *albicans* and *non-albicans Candida* species are indistinguishable. However, *non-albicans candida* species are often less susceptible to fluconazole than *C. albicans* and may require a greater dosage of antifungals to cure clinically (Chakrabarti et al., 2008). Early diagnosis of invasive fungal infections is important in the management of invasive fungal infection and in improving the outcome and preventing relapse of the underlying malignancy (Verma et al., 2003).

Previous studies have revealed blood stream infection rates ranging from 4 to 22% due to *Candida* species in cancer patients. The rise in incidence of non-*albicans*

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Table 1. Sequence and concentration of the primers, probe and probe specific T_m in real time PCR assays (Hebart et al., 2000).

Probre or primer	Sequence (concn)	Probe specific T_m (°C)
***Candida*-specific primers**	CCTGTTTGAGCGTCRTTT (0.15 µM) TCCTCCGCTTATTGATAT (0.5 µM)	
***Candida*-specific probe**		
C.alb-S	Cy5-CATTGCTTGCGGCGGTA-biotin (0.2 µM)	66 or 55
C.glab-S	Cy5GTTTTACCAACTCGGTGTTGAT-biotin (0.2 µM)	65
C.trop-S	Cy5-GGCCACCACAATTTATTTCA-biotin (0.2 µM)	63
C.rugosa-S	Cy5-CGAGCGAACTAGACTTT-biotin (0.2 µM)	60
C.para-S	Cy5-GAAAGGCGGAGTATAAAC-biotin (0.2 µM)	58

candidaemia and the emergence of antifungal resistance have further fuelled the need to carry out such a study (Halliday et al., 2005).

Azoles, antifungal agents have therapeutic activity against different *Candida* species. Among the azoles drugs, fluconazole shows that good tolerance has appeared and antifungal drug resistance is quickly becoming a major problem especially in immuno-compro-mised patients (Xu et al., 2000). This resistance also favors the emergence of *C. krusei* and *C. glabrata* (Cirak et al., 2003).

Conventional fungal identification methods may require 48 to 72 h or longer in order to yield definitive identifications, leading to significant morbidity and mortality that is associated with invasive *Candida* infections (Boyanton et al., 2008). Real time-polymerase chain reaction (PCR) technology can directly detect the presence of fungi in hours with high level of sensitivity and specificity *(*Chen et al., 2000; Loeffler et al., 2000).

The identification of species via melting curve analysis with species-specific hybridization probes further increases specificity. The fast turnaround time of less than 2 h is another advantage of the real-time PCR technology (Verweij and Meis, 2000). The aim of this study was to detect the incidence of *Candida* spp. in non-neutropenic cancer patients and evaluate the role of real time PCR in diagnosis of these cases.

MATERIALS AND METHODS

An approval was gotten from the ethical committee in Tanta Faculty of Medicine and a written consent from all participants in this study was performed over 12 months from March 2012 to March 2013 on cancer patients complaining of blood stream infection caused by *Candida* spp. (candidemia). An episode of candidaemia was defined as the isolation of *Candida* species from a single positive blood culture. 6 ml blood was taken from each patient; 3 ml were used for microbiological study using standard cultivation methods and 3 ml with citrate anticoagulant stored at −20°C until it was used for DNA extraction and real time-PCR.

Blood culture and organism identification

For diagnosis of candidaemia, 3 ml blood was collected in BacT/ALERT FA aerobic blood culture bottles (bioMérieux). Blood culture bottles were subcultured onto Sabouraud's dextrose agar. *Candida* cultures were considered positive if a 10 CFU appeared on the plate. Identification of these isolates was carried out by growth on the chromogenic medium CHROMagar by colony morphology and pigmentation according to the manufacturer's instructions and biochemical reactions (Forbes et al., 2002).

Real time-PCR analysis

From blood samples, DNA was extracted with a High Pure PCR template preparation kit by following the instructions of the manufacturer. DNA was eluted with 100 µl elution buffer and stored at -20°C until use **(**Hebart et al., 2000).

The 20 µl real-time PCR mixtures were prepared with 2 µl of LightCycler-FastStart DNA master SYBR green I (Roche Molecular Biochemicals, Mannheim, Germany), 4 mM $MgCl_2$, primers and probes as shown in Table 1, and 3 µl of DNA extract made up to 20 µl with water. PCR was performed in a LightCycler instrument with preliminary denaturation for 10 min at 95°C, followed by 60 amplification cycles (with a temperature transition rate of 20°C/s) of denaturation at 95°C for 8 s, annealing at 55°C for 10 s, and primer extension at 72°C for 10 s, with a single fluorescence acquisition step at the end of the extension. This was followed by a melting analysis of the probe-PCR product consisting of 95°C for 30 s and then cooling to 35°C for 60 s before the temperature was raised to 98°C at a rate of 0.2°C/s with continuous fluorescence acquisition. A final cooling step was performed at 40°C for 10 s. Samples were considered positive for *Candida* species upon the presence of a biprobe-specific melting peak. Specific T_m values of the biprobes are shown in Table 1.

RESULTS

During a 12 months period (from March 2012 to March 2013), out of 200 cancer patients with blood stream infection admitted to Oncology and Internal Medicine Departments in Tanta University Hospital, 89 were diagnosed with candidaemia were eligible for the study. 43 patients (48.3%) out of 89 were males and 46 (51.6%) were females, ranging in age from 15-79 (mean ± SD, 49 ± 3.49) years. Gastrointestinal and breast cancer were the most frequent among the studied group accounting for 35.9 and 26.9%, respectively. The mean of the hospital stay was 3.58 (range; 1-9) days. Tables 2 and 3 present demographic and clinical characteristics of cancer patients with candidaemia.

Table 2. Demographic characteristics of cancer patients with candidiasis.

Patient characteristic	Number (%)
sex	
Male	43 (48.3)
Female	46 (51.6)
Age (years)	
Range	15-79
mean±SD	49 ±3.49
Days admitted in hospital (days)	
Range	1-9
Mean±SD	3 ±4.45
Cancer type	
GIT	32 (35.9)
breast	24 (26.9)
lung	10 (11.23)
Head and neck	12 (13.48)
Others	11 (12.35)
total	89 (100)

Table 3. Clinical criteria of patients with candidaemia.

Patients with candidaemia	*C. albicans*	Non *C. albicans*
No. 89	No.15 (16.85%)	No.74 (83.15%)
Age (years)	36.07±17.4	34±17.02
Sex male/female	7/8	35/29
Steroid therapy	1 (6.66%)	15 (20.27%)
Broad spectrum AB	15 (100.00%)	74 (100.00%)
Mortality	5 (33.33%)	30 (40.54%)

Table 4. Frequency of isolation of *Candida* spp.from 89 cancer patients with candidaemia.

Candida spp.	Number (%)
Candida albicans	15 (16.9)
C.tropicalis	29 (32.6)
C. parapsilosis	21 (23.6)
C. rugosa	19 (21.4)
C. glabrata	5 (5.6)
Total	89 (100.00)

Considering the etiology of candidaemia, *Candida albicans* was the cause in 15/89 patients (16.9%) in contrast to non-*albicans Candida* species in 74/89 patients (83.1%). Of the non-*albicans* species, *Candida tropicalis* was responsible for the maximum number of episodes (29/89, 32.6%), followed by *Candida parapsilosis* (21/89, 23.6%), *Candida rugosa* (19/89, 21.4%) and *Candida glabrata* (5/89, 5.6%) (Table 4). The crude mortality in patients suffering from non-*albicans* and *albicans* candidaemia was 40.54 and 33.33%, respectively, the difference was not statistically significant. Distribution of *Candida* spp. in relation to cancer type is shown in Table 5.

As regard the results of real time-PCR, it was found that out of 89 blood samples which were positive for *Candida* spp. By conventional culture method 81 (91.01%) were positive for *Candida* spp. by real time-PCR and out of 111 negative blood samples by conventional culture methods 96 blood samples were negative for *candida spp.* by real time-PCR. Table 6 shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of real time-PCR when compared with conventional culture methods.

DISCUSSION

This study described bloodstream infections caused by *Candida* spp. in cancer patients using conventional methods

Table 5. Distribution of *Candida* spp. in relation to cancer type.

Diagnosis	*C. albicans*	*C. tropicalis*	*C. parapsilosis*	*C. rugosa*	*C. glabrata*
GIT	4 (4.49%)	7(7.86%)	5(5.61%)	3(3.37%)	1(1.12%)
breast	3 (3.37%)	7(7.86%)	2 (2.24%)	6(6.74%)	2(2.24%)
lung	1 (1.12%)	5 (5.61%)	3(3.37%)	4(4.49%)	-(00.00%)
Head and neck	6 (6.74%)	7(7.86%)	7(7.86%)	4(4.49%)	2(2.24%)
Others	1(1.12%)	3(3.37%)	4(4.49%)	2**(**2.24%)	- (00.00%)
Total 89 (100.00%)	15(16.9%)	29(32.6%)	21(23.6%)	19(21.4%)	55 (5.6%)

Table 6. Results of conventional culture and real time-PCR.

Sample	*BC results for *Candiada* spp.	PCR +ve	PCR -ve	Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Blood (*n*=200)	Positive (89)	81	8	89	91.01	95.6	94.18	92.30
	Negative (111)	5	96	111				

*BC: Blood culture.

and real time PCR to provide information on the epidemiology of candidaemia in this patient population by conventional culture methods; the results of this study showed that *Candida* spp. could be isolated from 89 (44.5%) cases out of 200 cases of blood stream infection from oncology unit in Tanta university hospital in a period of 10 months (from March 2012 to March 2012). These results are different from the results of Borzotta and Beardsley (1999) that reported the rate of candidaemia in cancer patients to be 1.5%. This difference may be attributed to the difference of the regimen of chemotherapy or the rules of infection controls in each institution.

In this study, non-*albicans Candida* species accounted for the majority of the episodes of candidaemia in 74/89 patients (83.1%). Previous studies have shown high rates of carriage of these species on the hands of healthcare workers *(*Rangel-Frausto et al., 2006). Thus, the hands of healthcare workers could be one possible source of transmission; these results are in agreement with the two studies of Chakrabarti et al. (2009) and Xess et al. (2007), respectively that could isolate *non-Candidal* strains from 75 and 66% population of patients. In the present study, *C. tropicalis* was isolated from 29 (32.6%) cases, these results came in agreement with the results of Pfaller et al. (2010), who reported that worldwide, the frequency of bloodstream infections due to *C. tropicalis* has increased. In India, *C. tropicalis* ranks first among non-*albicans Candida* species in causing candidaemia (Chakrabarti et al., 2009; Xess et al., 2007).

In this study, *C. glabrata* could be isolated from 5 (5.6%) cases and *C. krusei* was not detected in the isolated strains, the results are in accordance with that of Vollmer et al (2008) that could isolate only one strain of *C. krusei* from different clinical specimens in immunocompromised patients.

In the present study, *C. tropicalis* and *C. parapsilosis* were the two most commonly isolated species from cases of candidaemia. Thus, the species distribution in this study closely matched that of previous studies from North India (Xess et al., 2007). However, points in which this study differed from others were the lower isolation rate of *C. glabrata* (5.6%) and the absence of any case of candidaemia due to *C. krusei*. These findings probably reflect the absence of selective pressure exerted by azole antifungal prophylaxis at our institutions, which would have led to isolation of species that are known to be inherently resistant to fluconazole such as *C. krusei.*

In the present study, *C. rugosa*, could be isolated from a large number of cases of fungaemia (19/89, 21.4%), the result which is in agreement with that of Minces et al. (2009) who isolated *C. rugosa* from 25% of cases. Minces *e*t al. (2009) also was in agreement with our study in that blood stream infections with *Candida* spp. are especially common among patients in Intensive Care Units and associated with typical risk factors such as indwelling central vascular catheters, broad-spectrum antibiotics and steroid therapy. These results are in agreement with that of Colombo et al. (2006) who reported a large number of episodes of fungaemia due to *C. rugosa*. In addition, an outbreak of fungaemia due to *C. rugosa* was reported by Colombo et al. (2003).

Real-time PCR, is an approach that enables culture-independent screening of *Candida* infections within only a few hours. Rapid diagnosis would improve the survival rate in cancer patients. In this study, real-time PCR facilitated a rapid screening for simultaneous differentiation of the isolated 5 medically important *Candida* species causing blood stream infection in cancer patients in the same LightCycler run.

In the present study, real-time PCR showed mostly high

sensitivity. In some samples, PCR detected a greater number of different *Candida* species than culture. In these cases, patients had received antifungal therapy before sampling, which may be a reason for these discrepancies. Furthermore, whenever *C. albicans* was detected in addition to the cultured *Candida* (*C. glabrata* and *C. tropicalis*) species, *C. albicans* might have been overgrown by the other fungi and therefore missed by culture. On the other hand, culture detected a greater number of different species than real-time PCR in five samples, which have been already observed in previous studies (Willinger et al., 2003)

The present study revealed that blood culture is considered to be an important tool for the detection of systemic infection but it is time consuming and may lead to increase in the mortality of cancer patients with candidaemia. This is in contrast with Einsele et al. (1997) and Pfaller et al. (2010) who reported that the culture was positive in less than 50% of patients with disseminated candidiasis.

Conclusion

The high prevalence of non-*albicans* candidaemia should be considered prior to antimicrobical therapy in non-neutropenic cancer patients with candidaemia. Real-time PCR represents a rapid and accurate method for diagnosis of candidaemia.

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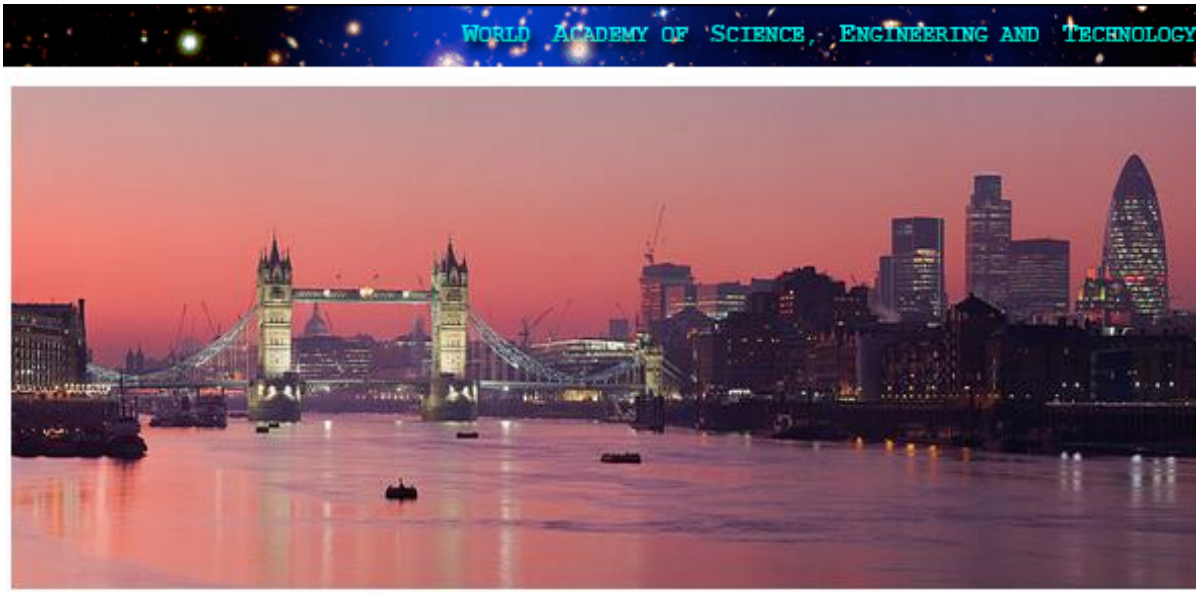

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