DISCUSSION

The diversity of life on earth has remunerated our nation with its bounty of nature enriched with variety of different types of ecosystem, that India is one among the world’s 15 nations that are exceptionally rich in species diversity (Erach, 2002). These natural ecosystems when carefully used sustain its survival but if misused, its productivity eventually decreases that finally disintegrates getting degraded. One such is the highly complex soil ecosystem who’s biotic and abiotic components support a teeming community of soil-dwelling fungi, bacteria, and microfauna and mesofauna. Owing to the abuse by human inventories in regard to increased industrialization activities, radical changes occur due to their pollutants affecting the soil edaphic characters as well as the microbiota with an impact on human health that on extremity can even lead to premature death (Saraswathy et al., 2010). However, optimistic approaches can be attributed towards these affected arenas by exploiting their microbiota for bioremediation and bioaugmentation strategies as well as for novel metabolite production studies.

The research plot investigated herewith tag along the same in exploiting the metabolite production of fungi isolated from diverse mycobiota of soil, being affected by distinguishing pollutants from industrial activities and also further mutation studies for enhancement of the metabolite. It is true that fungi rank as the most abundant microbe among the soil biota in terms of biomass and physiological activity fulfilling a range of important ecological functions (Kjoller and Struwe 1982; Schnurer et al., 1985). The problem of environmental pollution on account of essential industrial growth in terms of disposal of industrial waste (solid, liquid or gaseous) into the soil and limited studies on soil fungal community diversity forms the rationale of the present study to explore and exploit them in a constructive way.

To evaluate the diversity of fungi, four different soil types were chosen: one non – polluted soil (garden soil), three distinguish polluted soils – soil polluted by tannery waste (tannery soil), soil polluted by pharmaceutical waste (chemical soil) and soil polluted by plastic dump waste (plastic dumpsite). Initial studies in determining the physico – chemical characteristics of the different soils reveal the presence of heavy metals like chromium (Cr), lead (Pb), zinc (Zn) etc. that higher level of organic matter, potassium, zinc was present comparative
to non–polluted soil that highlights the presence of pollutants in the soil. The presence of heavy metals in these polluted soils like Cr, Pb, Zn in tannery soil and Zn in chemical soil with higher electrical Conductivity can alters the chelating properties of receiving water systems, which create conditions for free metal availability to flora and fauna. Also, the level of chromium in the tannery soil was at its permissible level for irrigatable field, yet it could influence upon the receiving biotic systems in longevity. Further, the higher level of organic matter can also act upon the bio-availability of heavy metals. Higher level of Zinc and Potassium can result in their hyperaccumulation in the biota. Presence of Zn at higher concentrations can retard the growth and development of plants in the nearby crop fields by interfering with certain important metabolic processes. Prolonged persistence of these heavy metals can also enter the ground water systems in turn could pose serious threats to ill–health of human, that can even be mortal.

Supportive evidences could be obtained from the reports of Chandramohan and Bharati (2009) that an irreversible damage has been caused in Pallikarani due to the leachates from plastic dumpsite which had seeped into subsurface and entered recharge channels through which groundwater entered the domestic and deep bore wells. The groundwater quality has deterioriated which could be visibly seen with a pungent foul smell, reddish to orange in color, as reported by the residents near Pallikaranai Marsh. Also, this extends to the Buckingham canal that meander these polluted subsurface waters resulting in death of fishes in Kovalam creek. Jayaprakash et al., 2010 has also indicated that the marshy region is more heavily contaminated with Cd, Hg, Cr, Cu, Ni, Pb, and Zn than other regions on the southeast coast of India. A recent study has also revealed the dominance of heavy metals present in Pallikaranai wetland following the sequence: Pb>Cr>Fe>Ni>Zn>Cd>Cu (Ramachandran et al., 2012). In addition, the presence of heavy metals like lead, cadmium, zinc, cobalt, chromium etc. in the environment associated with industrial areas of Ranipet and Vellore are well accounted by many research papers (Mahesh and Selvaraj, 2008; Gowd and Govil, 2008, Saraswathy et al., 2010, Ambiga and Annadurai, 2013) with a remark on the effects of lead toxicity leading to impairment of kidney functioning prone to children rather than adults, biopersistence of cadmium leading to weakening of bones, kidney malfunction, circulatory disorders and other heavy metals resulting in occupational diseases such as asthma, chromium ulcers and skin diseases. Similarly, the effect of pharmaceutical drug effluents contaminating the local ecosystems can end up in the occurrence of multidrug resistant pathogens which affects aquatic life of receiving water systems, that clinical studies on gene
expression in a fish affected by pharmaceutical effluent in Patancheru indicates sublethal toxicity posing threat to humans for impaired liver and kidney function (Larsson et al., 2007; Gunnarsson et al., 2009).

These pollutants do also affect the microbiota that is evidently seen via fungal biodiversity studied in these polluted soil types. In general, all major taxonomic groups of fungi are encountered in soils (Bills et al., 2004). Likewise the non-polluted garden soil harbored almost major groups of fungal species with 502 isolates, belonging to several classes of zygomycetes, ascomycetes and hyphomycetes, showing high species richness and high species diversity as indicated by the Simpson’s diversity index of D = 0.05. However, comparative differences in the presence of less diverse fungal species in the polluted soils explains the selection pressure exerted by the pollutants, otherwise the toxic metals and chemicals on the soil fungal population that can even result in development of multi metal/drug resistant fungi in longevity. Particularly, alkaline chemical soil revealed less fungal species indicating the notorious nature of the tarnished pharmaceutical effluent contaminating the soil ecosystem. Yet, plastic dumpsite harbored nearly 31 fungal species with 16 genera comparable to the garden soil. According to the literature, neutral soils enhance the growth of bacteria, while acidic soils enhance the growth of fungi (Meysami and Baheri, 2003). This could explain the occurrence and distribution of fungi in acidic/alkaline polluted soil samples.

Statistical analysis also showed significant finding (P<0.001) which envisaged that the diversity of fungi is affected by the persistence of different pollutants treating different soil types. Similar kind of results were obtained by Ahmad et al., 2005 while studying the heavy metal biosorption potential of Aspergillus and Rhizopus sp. isolated from wastewater treated soil. Supplementary reasons like pH of polluted soil ranging from acidic to alkaline, moisture content, presence of organic matter, changes in soil edaphic character turning infertile etc. owing to the persistence of pollutants can also act upon the frequency and distribution of mycoflora resulting in higher dominance of selected fungal species. Related studies do report that physico chemical properties of the soil affect the density and diversity of microbes in the soil. The moisture content in soil acts as solvent and is essential for microbial functioning. A certain minimum level of organic matter and moisture content is essential to ensure the presence of an active microbial population in the soil (Rohila and Salar, 2012). Excessive moisture leads to inadequate oxygen
diffusion thereby affects fungal colonisation in soil. A lower fungus diversity in more polluted soils provides evidence that pollution may reduce the suitability of that soil as a habitat for specific groups of fungi, such as the genera *Cladosporium*, *Scopulariopsis*, *Geotrichum*, *Paecilomyces*, *Myrothecium*, *Mortierella*, *Phoma* and some species of the *Penicillium* and *Trichoderma* genera. Other fungi (*Curvularia*, *Penicillium*, *Fusarium*, *Rhizopus*, and sterile mycelia forms) were found in general abundance in the polluted soils. Similar kind of results were obtained by Pec et al., 2009 from soils of deciduous forests affected by long term industrial pollution showing minimal fungal diversity.

Comparing with polluted soil isolates, the frequency and relative density of most fungi was higher in garden soil. However, *Aspergillus* sp., was found higher in polluted soil with its frequency and relative density pursuing the order of *A. niger* > *A. terreus* > *A. fumigatus*, the order being reverse in garden soil. Thornton (1956) had proposed this arranging order of fungal species isolated from the soil as the “soil fungal pattern”, that are expected to vary from one soil to another. Further, the relative dominance studies showed Aspergillus to be the only highly dominant genera in all the soil types, particularly in polluted soils with high - different speciation as observed in plastic dumpsite soil. Particularly *A. terreus* appeared dominant in all the four types of soils, followed by *A. niger* > *A. fumigatus* proving the high adaptability of that meticulous species. Certain soil fungus species are better adapted to the disturbance produced by the addition of metals would overcome the stress situation and complete their life cycles (Iram et al., 2011). Reviews suggest that major differences among the species suggest that fungi follow different strategies to establish symbiosis and probably Aspergillus sp., was preferentially found in soil samples with the differences in functioning (Johnson et al., 1992; Allen et al., 1995; Bever et al., 1996). The relationship between genetic diversity within populations and heavy-metal stress in soils may lead to an increase in diversity with a moderate metal loading, followed by a sharp decrease at higher levels of stress (Giller et al., 1998). And this could explain the reason for minimal fungal population present in the chemical soil with least occurrence, distribution and diversity of fungi.

Isolation of soil saprophytic fungi not only depends on the media, but also on the technique chosen that traditional methods rely on soil plating method and soil dilution or suspension plating method. However variation of these techniques like particle filtration or soil
washing, direct hyphal isolation, direct comparison method etc. are more appropriate for isolation of basidiomycetes and certain soil invertebrates (Davet and Rouxel 1997). Although warcup’s soil plating method yields isolation of active mycelia, the soil dilution technique is the best-known method for the estimation of microorganisms distributed in soil. Soil dilution method is a combination of gentle dispersion, soil dilution and serial dilution that also aids in the distribution and growth of disseminated and inactive spores, spores and hyphal segments into the media (Zakaria et al., 2010). Further, choosing an isolating medium is of paramount importance in isolation of fungi and that Potato dextrose agar has been widely used as a relatively rich medium for growing wide range of fungi.

Spectrophotometric assay methods based on turbidimetric measurements have been used in a small number of mycological growth studies where fungal biomass is correlated with optical density for monitoring fungal growth over time (Rodrigues et al., 2009). Initially there is a delay before the optical density increased due to fungal growth referred to as the lag phase which was shown to be due to germination of the spores and initial hyphal formation (Meletiadis et al., 2001). The growth of A. terreus reached its maximum on 10th day with an O.D of 3.25 during log phase. The growth of filamentous fungi is usually characterized by smoother curves and long transition periods that are being evident for A. terreus too. However the growth of filamentous fungi is also dependent on the medium and environmental conditions employed. An optimal nutrient medium should provide not simply adequate growth but the best possible growth in order to allow molds to grow without restriction and express all phenotypes. Different types of media, pH and temperature conditions were studied that revealed changes in turbidometric measurements and the growth curves among different strains of A. terreus. The media components and the culture conditions affects the germination and elongation rates of spores and hence the growth rates. Review also states that the lag phases are species dependent with small inter strain variation, thus their growth curves and are highly reproducible. Similar studies conducted reveals Yeast Nitrogen Base medium and Sabouraud broth were observed to be highly nutritious media providing the highest growth for R. microsporus and A. fumigatus with growth rates three to four times higher than those achieved in RPMI media (Meletiadis et al., 2001). Comparison of media types and culture conditions and checking for corresponding growth rates also aid us to optimize the media for standardization for yielding higher biomass concentrations.
In response to their surroundings, the fungal exo-metabolome and endo-metabolome consisting of secondary metabolites and primary metabolites produce them as a reaction to the biotic and abiotic environment. These secondary metabolites are extracted by organic solvents and separated whose profiling has been used quite extensively for taxonomic purposes. The secondary metabolites extracted from mycelial mat and culture filtrate of different strains of *A. terreus* using different organic solvents yielded varied quality of chemical compounds like alkaloids, flavonoids etc. explains their qualitative presence as well as the polarity of the organic solvent used to obtain metabolites of hydrophilic and hydrophobic nature (Belofsky *et al.*, 1998; Holler *et al.*, 2000 and Lin *et al.*, 2000). Similarly secondary metabolites have been extracted from various other fungi like *Alternaria* sp., *Chaetomium* sp., *Curvularia* sp., etc. using various solvents like methanol, hexane, dichloromethane, ethyl acetate and butanol tested for potency via antimicrobial, antileishmanial, anticancer and antioxidant studies. Extracts from hexane, acetone, dichloromethane, and methanol were more effective indicating their potency in efficient extraction of biologically active metabolites present in those saprophytic fungi. Similarly in our results hexane and acetone were better solvents in extraction of various metabolites. Given the chemical nature of such small organic molecules they can be detected by different spectroscopic tools such as IR, UV, FLD, MS etc. (Nielsen *et al.*, 2004). These organic extracts following UV and IR spectral analysis suggest the presence of various compounds evident through their absorption spectra and chemical bonds. IR spectra infers the physical properties of the active compounds that the absorption peaks indicate C-H stretching for alkanes, C=O stretching for ketones, asymmetric nitro groups, etc. that in particular the existence of absorption peaks 1683, 1684, 1688 indicates C=C stretching vibration reveals the presence of β Unsaturated amines bonds which can be related to the presence of unsaturated fatty acids in the organic extract. Also, the peaks at 3005 cm⁻¹ can be attributed to the trans-CH=CH-groups, 1455 cm⁻¹ and 1373 cm⁻¹ for the asymmetric and symmetric deformation modes of the C-H group, 965 cm⁻¹ for trans conjugated alkene -CH=CH- indicating the presence of the β-carotene in the extracts. Concurrent evidences on UV and FT –IR reports could be obtained from various studies of Ammawath and CheMan (2010), Bunghez and Mariana (2011), Moha *et al.*, (1999), Bertran *et al.*, (1999), Sinclair *et al.*, (1952) indicating the presence of β - carotene and fatty acids in the extracts.
Quantitative profiles of fatty acids (Blomquist et al., 1998), the pattern of utilization of C- and N- sources, temperature, water activity, pH, atmosphere, redox relationships, etc. aids in the characterization of different filamentous fungi (Andersen and Frisvad, 2002). Also, fatty acid production from the fungal mycelial mat is almost directly proportional to the biomass production. Changes in the lipid constituents of *Penicillium atrovenetum* were analyzed by Etten and Gottlieb (1965) during their growth and development that the total fatty acids increased to a maximum during the log phase and the major fatty acids were Palmitic, Stearic, Oleic and Linoleic. Also, lower percentage (on the basis of total fatty acids) of linoleic acid were produced by the younger mycelium compared to the ungerminated spores. Results obtained in the present study also infers that optimization of the media conditions in standardizing for higher biomass yields have proven the fact that higher the biomass, higher was the percentage of free fatty acids and hence the acid value. Higher yield of nearly 1.21g of biomass per 100 ml of culture media were obtained with the AT–PAL cultures grown in PDB under conditions of pH 6 and room temperature, with 12 h cycle of light and dark conditions yielding 42.5, 37.09 and 35.43 percentage of free fatty acids with an acid number of 84.69, 73.81 and 70.51 compared to other cultures respectively. Further, the fatty acid profiles also differ qualitatively and quantitatively among the different strains of *A. terreus* that it has been stated that cellular fatty acid profiles can be used to differentiate and identify genera, species and strains of yeasts and filamentous fungi (Stahl and Klug, 1996). Synthesis of unsaturated fatty acids in the spores and the mycelium of the thermotolerant and thermophilic fungi of order mucorales was increased by lowering the growth temperature from 48°C to 25°C as studied by Sumner and Morgan (1969). Optimization of the cultural conditions in *Penicillium breviconactum* favoring production of the biologically active compounds (*i.e.*, medium, incubation period, rpm, temperature and pH value) resulted Malt Extract with peptone as suitable substrates for metabolite production. Similarly, optimal conditions of 30°C, 200 rpm for 7 days yielded maximum dry biomass and GLA, respectively in an oleaginous fungal strain of order mucorales (Ahmaed et al., 2006). Analogous results yielding highest dry weights (11–12 g/l) and lipid contents (∼24%, w/w) were observed when glucose or fructose was used as carbon source whereas the highest amount of γ-linolenic acid (∼26%) was determined in starch-grown cells of *Mortierella* sp (Hansson and Dostalek, 1988). Using gas chromatography technique, these volatile fatty acid metabolites can be separated and detected that fatty acid profiling finds various applications like estimation of fungal biomass via signature
fatty acids, multivariate discriminant analysis among the species at intra specific level etc. Relative concentration as well as the type of each fatty acid present are useful characteristics for separating taxa of varied filamentous fungi, including Oomycetes, Zygomycetes, Basidiomycetes and even sterile mycelia that have emerged as recent developments in fungal taxonomy (Guarro et al., 1999).

Not only fatty acids, but also the carotenoids play an important role that microorganisms accumulate several types of carotenoids as a part of their response to various environmental stresses. Light, temperature and culture medium composition have direct influence in carotenoids production. Effect of culture media, pH and temperature on different strains of A. terreus yielded varying production of β-carotene levels, reproducing the results of analogous studies made by Papp et al., 2013 on different strains of M. circinelloides and B. trispora. In addition two different mechanisms were postulated via enzyme aggregate hypothesis indicating the organization of carotenogenic enzymes during the exponential phase as well as in later part of growth cycle for the production of β-carotene in P. blakesleeanus (Ootaki et al., 1973). Higher yield of nearly 1.21g of biomass per 100 ml of culture media were obtained with the AT–PAL cultures grown in PDB under conditions of pH 6 and room temperature, with 12 h cycle of light and dark conditions yielding 34 µg/ml, 30 µg/ml and 28 µg/ml of β-carotene compared to other cultures respectively. It has been researched that optimum temperature for β-carotene production is 25°C and highly synthesized in well formed mats of mycelia, differing with varying pH levels of media. Also, organization of carotenoids into lipid bilayer of cell wall or spherosomes or lipophilic layer of ER indirectly paves the importance of biomass yield for carotenoid production (Bennet and Siegler, 1983). Qualitative and quantitative analysis of carotenoids have also been used for taxonomic classification of pigment producing fungi such as e.g. Rhodotorula sp.,. Research on carotenoid production has been reported in different fungal classes; zygomycetes (e.g., P. blakesleeanus), ascomycetes (e.g., N. crassa and Gibberella fujikuroi), and basidiomycetes X. dendrorhous).

It has been stated that, genetic modification approaches could be used via mutation studies that can aid in generation of productive species. To improve ARA productivity by M. alpina, approaches like genetic modifications have been used to produce highly productive species (Zhu et al., 2006). Even MNNG have been used for producing stable high yielding mutants producing increased level of ω-1-hydroxy fatty acids (Wu et al., 2008). Likewise, four
mutagens - UV radiation, benomyl, ethyl methanesulfonate and EtBr were employed in generating astaxanthin-hyperproducing strains of the yeast X. dendrorhous DSM 5626 (Stachowiak, 2013). The present investigation does emphasize the use of two mutagens: EtBr and mosquito repellent that has enhanced the simultaneous increase of both fatty acids as well as β-carotene. Use of mosquito repellent as an emerging mutagen has been reviewed by Rasool and Mushtaq (1991). Further, use of chemical mutagens like MNNG and EMS has been studied in several organisms like E. nigrum, Rhodotorula, Phaffia rhodozyma etc. that has proven successful for higher productivity of β-carotene (Ueno et al., 2012). The underlying mechanism of mutation lies in alteration of gene function to yield oxidaseless strains thereby preventing the oxidation of β-carotene to astaxanthin, thereby accumulating higher levels of β-carotene. Presently, a 22 fold increase in Pentadecenoic acid of 29.29g/100g, 29 fold increase in the production of Palmetoleic acid of 28.96g/100g and novel production of Myristoleic acid of 21.57g/100g from the mutant ATSMC – 123, also yielding 48.72 mg/l of β-carotene inter relate the simultaneous production of both fatty acids and β-carotene that are inter competing for their production originating from Acetyl CoA substrate. It is through fatty acid synthesis pathway and mevalonate pathway the fatty acids and carotenes are produced from the Acetyl CoA substrate.

Role of β-carotene as anticancer and antioxidant agent as well as the apoptotic death of cancer cells are inter related that it is one of the most efficient substances known for quenching the excitation energy of singlet oxygen and for trapping certain organic free radicals. The IC50 value of β-carotene obtained from the mutant strain AT SMC – 123 was found to be 125 μg/ml against both MCF-7 and Hep–G2 cell lines causing apoptotic fragmentation of DNA in cancer cells with 50.2% radical scavenging activity. It is stated that β-carotene enhance gap junctional communication and inhibit lipid peroxidation in chemically induced neoplastic transformed cells and also as a chain breaking anti-oxidant in the lipid phase by neutralizing peroxy radicals controlling cancer growth (Figure 1). This entails their possible mechanism of action where β-carotene is converted into 8/, 10/ and 12/-apo carotenals, further oxidized into isomers of retinoic acid that forms homo (RXR/RXR) or hetero (RXR/RAR) dimeric proteins binding with RXRE or RARE sites of DNA sequences, making it inaccessible thereby modulating the transcriptional machinery of regulatory proteins. This further leads to TR3/RXR-α heterodimer formation in the nucleus whose subsequent translocation in the cytoplasm down regulate antiapoptotic protein
like Bcl-2 and Bcl-xl inducing apoptotic protein Bax resulting in ATRA induced apoptosis in the cancerous cells (Mukherjee et al., 2011).

Thus the potency of β - carotene gains further attention to insight its molecular aspects of genes responsible for their synthetic pathways in the high yielding mutant strain AT SMC – 123 apart from its quantitative production yield. It is evident from the studies of Velayos et al., 2000 that a single carp gene codes for a protein with two different enzymatic activities, lycopene cyclase and phytoene synthase responsible for β - carotene synthesis, which are encoded by independent genes in organisms other than fungi. Initially genomic DNA was extracted from the mother culture and mutant AT SMC – 123 followed by PCR amplification using degenerate primers to isolate phytoene synthase gene. However it resulted in multiple bands indicative of binding of degenerative primers with the DNA apart from the specified locus. Yet PS Primer 1 produced the amplified products of expected size of approx. 555 bp and therefore can further be employed in future for PCR optimization and gene sequencing studies. Similar studies have reported a gene homologous to al-2 and carRP gene – called as the crtYB gene from the basidiomycetous yeast X. dendrorhous [Verdoes et al., 1999]. This gene is denoted as `phytoene b-carotene synthase', that is, the same as phytoene synthase/lycopene cyclase. Althought the phytoene synthase-encoding genes are well conserved among very different organisms, from bacteria to plants and distinct from the lycopene cyclase, the fungal gene encoding both activities seems to have evolved by fusion of previously independent genes (Velayos et al., 2000).

On the whole the present investigation emphasize the optimistic approach of mutating soil isolates from polluted environment for increased production of fatty acids like palmetoleic acid, myristoleic acid and β - carotene which finds applications in therapeutics, clinical and industrial arenas. Use of mosquito coil as a mutating agent as well as mutation of soil fungal isolate – A. terreus for productive yield of fatty acid and β - carotene makes the study novel that can further be explored in various strategies of molecular concepts, gene expression studies, drug designing arenas of bioinformatics and so on. Fatty acids and β - carotene obtained can also be subjected further for absolute purification and scaled up for industrial production. Novel production of myristoleic acid, an uncommon ω – 5 fatty acid, clinically proven for its anti pancreatic cancer effects, anti inflammatory role etc. scores a significant remark from the present investigation of mutant studies. In addition, the β - carotene thus obtained from a novel source of
a mutant soil fungal isolate – *A. terreus* has proven for its potency as anti cancer and anti oxidant agent inducing apoptotic DNA fragmentation in cancer cells that can farther be analysed into molecular level for better understanding and clear concepts. Altogether the study promotes a novel proposal in initiating the production of β-carotene and uncommon myristoleic fatty acid from mutant strains of *A. terreus* isolated from soils of distinguishly polluted sites.