CHAPTER 2 LITERATURE REVIEW

2.1 Endophytic fungi and plant interaction

Endophytic fungi are fungal microorganisms, which spend the whole or part of their life cycle in either intercellular or intracellular space of the healthy tissues of the host plant (Saikkonen *et al*., 1998). Fungi live within the plant without symptoms of diseases. There is little information about asymptomatic feature of endophytes. It was only known that the asymptomatic endophytic fungus is unable to produce a toxic metabolite which the pathogenic strains produce. Therefore, the endophyte is unable to induce disease in the host plant (Abang *et al*., 2009). They are found in almost all kinds of plants, including grasses, trees and herbaceous plants. Endophytes are considered plant mutualists. They receive nutrition and protection from the host plants while the host plants benefit competitive abilities and increased resistance to herbivores, pathogens, and various biotic stresses conferred by endophytes (Carroll, 1988).

Some fungal endophyte species infect one or a few hosts (endophytes with specific or limited host range) while others infect several hosts (generalist endophytes). Most of these endophytes establish localized infections (Stone, 1986). Studies, conducted on temperate broad leaf and particularly in evergreen tropical rain forests, suggest that the colonization of plant tissues by endophytes involves several steps including host recognition, spore germination, penetration of the epidermis and tissue colonization (Gao *et al*., 2010). After endophytes are successfully colonized host tissue, the endophytic niche occurs. In the endophytic niche, endophytes obtain a reliable source of nutrition. The organic nutrients in the intercellular spaces, endophytic niches, consist of sugar, sugar alcohol, amino acids, organic acids, and inorganic ions such as potassium, calcium, sulphur, phosphorus, and chloride. The fungal endophyte colonization goes usually from undetectable or low levels in very young tissues such as recently emerged leaves and shoots to high levels in mature tissues and to full colonization in older ones. A nutrient-rich organ is responsible for distribution of fungal hyphae (Kuldau and Bacon, 2008).

Endophytes are the subject of intensive research because of the potential that they hold in agriculture as a source of beneficial effects to their host plants, such as increased vigor and tolerance to a range of abiotic (e.g., drought and heavy metals) and biotic stresses through production of mycotoxins or other fungal derived molecules (Kuldau and Bacon, 2008; Clay, 1998). In particular, some field trials have shown that inoculation with particular endophyte strains can benefit their hosts by limiting the damage by pests or reducing the dispersal capacities of their pathogens (Miller *et al*., 2002).

In particular, fungal endophytes have been reported from all plant species surveyed, including representatives from all ecosystems. In addition, these fungi can be isolated from different plant organs and tissues, including roots, stems, branches, leaves, flowers, and fruits (Saikkonen *et al*., 1998; Stone *et al*., 2000, 2004; Arnold, 2007). Commonly found fungal endophytes (excluding mycorrhizal associations) belong to diverse classes of Ascomycota, mostly Dothidiomycetes, Leotiomycetes, and Sordariomycetes, although Basidiomycota endophytes have been observed to be common in some hosts (Stone *et al*., 2004; Crozier *et al*., 2006; Sieber, 2007).

2.2 Identification of endophytic fungi

About 64,000 fungal species were known by 1983, and since then about 1,500 species have been described and named as newly discovered each year. However, it is usual that about 60% of such discoveries are fungi that have already been found and described under a different name. Hence the number of fungi known is probably increasing at about 600 species a year. The correct identification of fungi is of great practical importance not only in a clinical setting but also in plants association, biotechnology, and environmental studies. An enormous number of species of fungi are already known, and taxonomists are being kept very busy with recognizing and describing new species and grouping taxa. The classification of many fungi is based mainly on morphology (Carlile and Watkinson, 1994).

2.2.1 Morphological identification

Fungal taxonomy is traditionally based on comparative morphological features (Lodge *et al.*, 1996; Sette *et al.*, 2006). The identification system of fungi relies principally on observable characteristics and the fungal life cycle. This is the phenotypic approach requiring morphological characteristics of mycelium, spore formation and shape of spores that are the most important elements for distinguishing morphological species. Morphological species can be based on colony surface textures, hyphal pigments, exudates, margin shapes, growth rates, and sporulating structure (Redlin and Carris, 1997). However, special caution should be taken when closely related or morphologically similar endophytes are identified because the morphological characteristics of some fungi are medium dependent and cultural conditions can substantially affect vegetative and sexual compatibility (Zhang and Gao, 2006; Hyde and Soytong, 2007). Furthermore, the conventional methods cannot be applied for identifying fungal isolates that fail to sporulate in culture, which are categorized as *Mycelia sterilia*. Various optimizations of growth conditions have been used to promote sporulation of these fungi, such as different culture media, potato dextrose agar (PDA), malt extract agar (MEA), corn meal agar (CMA), potato carrot agar (PCA), and water agar (WA), as well as the inclusion of host tissues in plate cultures. Nevertheless, a large number of fungi still do not sporulate in culture, and these *Mycelia sterilia* are considerably frequent in the endophyte studies (Lacap *et al*., 2003).

2.2.2 Molecular based identification

The molecular techniques are of high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains over a wide range of taxonomic levels (Sette *et al*., 2006). The molecular methods offer three main advantages over morphological methods: (i) some can be directed at strain level or at least subspecific-level identification; (ii) they require less time than morphological methods; and (iii) large numbers of samples can be dealt with more easily.

Many filamentous fungi have been detected and identified by comparative analyses of the rRNA genes (rDNA) sequences. Numerous sequences of rRNA genes have been obtained primarily by isolating and sequencing individual cloned genes; however, they were sufficiently difficult and laborious that the study of large numbers of species or individuals required exceptional efforts. Direct rRNA sequencing has also been used to rapidly obtain sequence data; however, this method requires relatively large amounts of RNA and is prone to errors because only one strand is sequenced. This results in a relatively high frequency of errors (1-5%) because ambiguities cannot be resolved by comparison with the opposite strand. Another potential problem is that RNA sequences of some organisms have extensive post transcriptional sequence modifications or editing and do not reflect the actual DNA sequences of the genes. Since the polymerase chain reaction (PCR) was developed by Mullis (1983), direct sequencing of amplified DNA fragments offer several advantages over cloning and direct rRNA sequencing: (i) the method utilizes relatively crude preparation of total DNA; (ii) only small amounts of DNA are required, about 0.1 to 10 ng per amplification; (iii) both strands of the gene can be sequenced, which reduces error; (iv) the method is compatible with automated DNA sequencing instruments; and (v) the regions other than nuclear rRNA genes are accessible for sequence analysis. Universal primer sequences for both nuclear and mitochondrial rRNA genes as well as the internal transcribed spacer (ITS) have been described for fungi.

The fungal nuclear rRNA genes are arranged as tandem repeats with several hundred copies per genome. Each repeated units consist of 18S, 5.8S, and 28S rRNAs, two internal transcribed spacers (ITS1 and ITS2) and two external transcribed spacer sequences (5´and 3´ ETS). These units are transcribed by RNA polymerase I and separated by non-transcribed intergenic spacers (IGS) as represented on Figure 2.1 (Bruns *et al*., 1992). Sequences of 18S rDNAs are conserved and have been used in phylogenetic analyses of fungi of higher taxonomic ranks such as classes or orders (Swann and Taylor 1993, Wilmotte *et al*., 1993). On the other hand, ITS rDNAs are so variable that they often cannot be aligned accurately between genera and are now commonly used in the identification of species within a genus. Mitochondrial small subunit (mt SSU) rDNAs were reported to evolve 16 times faster than 18S rDNAs (Bruns and Szaro, 1992), but are less variable than ITS rDNAs. Nearly full-length sequences of 18S and ITS rDNAs for fungi can be amplified by PCR, but only partial sequences of mt SSU rDNAs have been amplified (Hibbett and Donoghue, 1995). For this reason, mt SSU rDNA sequences have not been popular among molecular systematists, and phylogenetic investigation of fungi based on partial sequences of mt SSU rDNA has been found unsatisfactory.

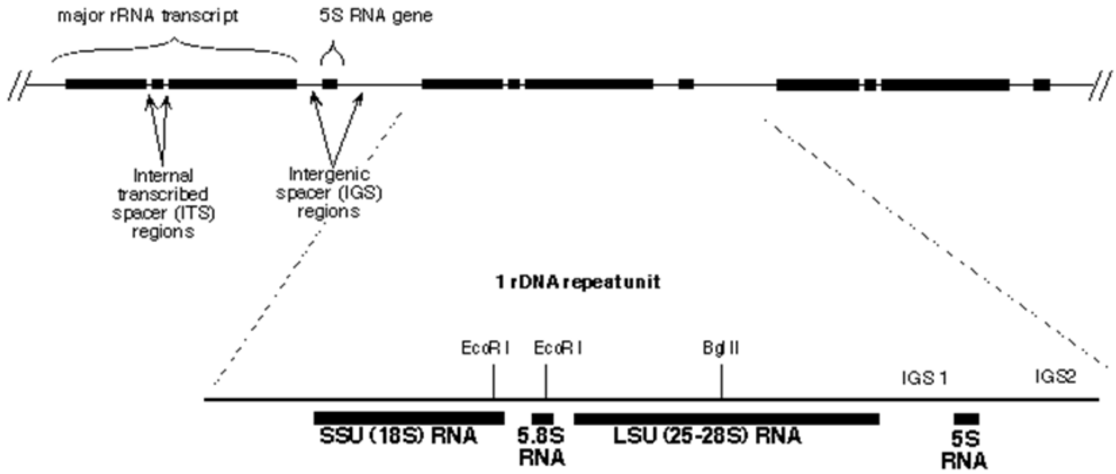

Figure 2.1 Fungal nuclear ribosomal RNA

(http://www.biology.duke.edu/fungi/mycolab/primers.htm)

The ITS region is now perhaps the most widely sequenced DNA region in fungi. Several features make it a convenient target region for molecular identification of fungi: (i) in fungi, the entire ITS region is often between 500 and 800 bp and can be readily amplified with “universal primers” that are complementary to sequences within the rRNA genes (White *et al*., 1990) given that several taxon-specific primers have been described allowing selective amplification of fungal sequences (Figure 2.2, Table 2.1), (ii) the multicopy nature of rDNA repeat makes the ITS region easy to amplify from small, dilute, or highly degraded DNA samples, and (iii) several studies have demonstrated that the ITS region is often highly variable among morphologically distinct fungal species (Gardes and Bruns, 1991).

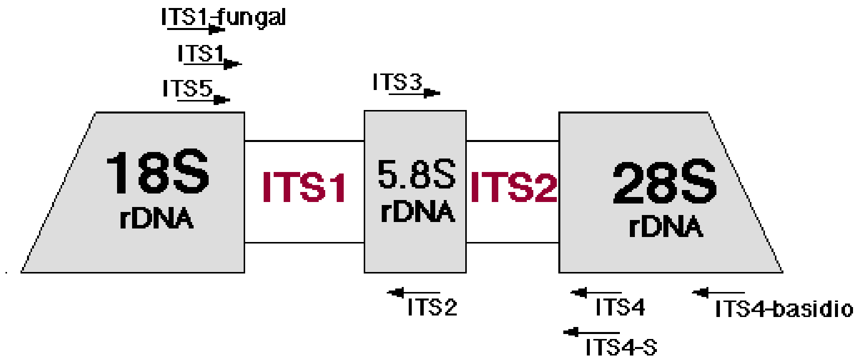

Figure 2.2 Internal transcribed spacer (ITS) region primers map (http://nature.berkeley.edu/brunslab/)

Several recent studies have shown that ITS regions can be successfully used in the studies of fungi. Guo *et al*. (2000) identified *Mycelia sterilia* from *Livistona chinensis* using rDNA ITS sequence analysis. Peintner *et al*. (2003) first recorded ectomycorrhizal *Cortinarius* species from tropical India and established their phylogenetic position using ITS sequences. Ganley *et al*. (2004) studied morphologically similar endophytes and parasites based on the ITS regions, and found that the endophytic fungi in west white pine were actually most closely related to, but distinct from, the parasites. Sette *et al*. (2006) identified endophytic fungi from coffee plants at least at the genus level, and the results were in accordance with the previous morphological characterization. According to Lin *et al*. (2007), 48.9% of the non-sporulating fungi from *Camptotheca acuminate* were identified based on rDNA ITS sequences analysis.

Table 2.1 Primers for amplification of fungal ribosomal RNA genes

rRNA	**Primer Sequence (5'→ 3')**
Nuclear, ITS	
ITS1	TCCGTAGGTGAACCTGCGG
ITS2	GCTGCGTTCTTCATCGATGC
ITS3	GCATCGATGAAGAACGCAGC
ITS4	TCCTCCGCTTATTGATATGC
ITS5	GGAAGTAAAAGTCGTAACAAGG
Nuclear, small subunit	
NS1	GTAGTCATATGCTTGTCTC
NS2	GGCTGCTGGCACCAGACTTGC
NS3	GCAAGTCTGGTGCCAGCAGCC
NS4	CTTCCGTCAATTCCTTTAAG
NS5	AACTTAAAGGAATTGACGGAAG
NS6	GCATCACAGACCTGTTATTGCCTC
NS7	GAGGCAATAACAGGTCTGTGATGC
NS8	TCCGCAGGTTCACCTACGGA
Mitochondrial, small subunit	
MS1	CAGCAGTCAAGAATATTAGTCAATG
MS2	GCGGATTATCGAATTAAATAAC
Mitochondrial, large subunit	
ML1	GTACTTTTGCATAATGGGTCAGC
ML2	TATGTTTCGTAGAAAACCAGC
ML3	GCTGGTTTTCTACGAAACATATTTAAG
ML4	GAGGATAATTTGCCGAGTTCC
ML5	CTCGGCAAATTATCCTCATAAG
ML6	CAGTAGAAGCTGCATAGGGTC
ML7	GACCCTATGCAGCTTCTACTG
ML8	TTATCCCTAGCGTAACTTTTATC

2.3 The *Nodulisporium* sp. as producers of bioactive compounds

The genus *Nodulisporium* was reported as anamorph of several genera of Xylariaceae, e.g., *Hypoxylon* Bull, *Xylaria* Hill: Schrank, *Camillea* Fr., *Biscogniauxia* Kuntze, *Anthostomella* Sacc., *Daldinia* Ces. & De Not., *Entonaema* A. Moller, and *Rhopalostroma* D. Hawks (Eriksson and Hawksworth, 1993). These teleomorph genera are distributed worldwide and known to be plant pathogens, wood decay fungi, lichens and even endophytic fungi (Whalley, 1996; Li *et al*., 2007; Wang *et al*., 2005).

The endohytic fungus *Nodulisporium* has been observed in a wide range of hosts including trees and herbaceous plants (Polishook *et al*., 2001; Wu *et al*., 2010). Moreover, many species of the genus *Noduliosporium* have been involved in causing allergic fungal sinusitis in human (Cox *et al*., 1994). The classification of *Nodulisporium* was provided as the following:

Classification

Direct Parent	mitosporic Xylariaceae
Kingdom	Fungi
Phylum	Ascomycota
Class	Sordariomycetes
Order	Xylariales
Family	Xylariaceae
Genus	*Nodulisporium*

This genus has been reported as a producer of diverse class of secondary metabolites. The bioactive secondary metabolites produced by *Nodulisporium* sp. with their potential applications in medicine, industry and agriculture were described in this section.

2.3.1 Insecticidal compounds

The *Nodulisporium* fungal species are known to produce nodulisporic acid A as a potent, long-lasting, nontoxic systemic orally-active agent that kills fleas on dogs and is economically important as a good substitute of the existing insecticides (Narzir *et al*., 2012). The nodulisporic compounds were first isolated from an endophyte *Nodulisporium* sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for more *Nodulisporium* sp. or other producers of more-potent nodulisporic acid analogues. Nodulisporic acids (indole diterpenes), exhibits potent insecticidal properties against the larvae of the blowfly, by activating insect glutamate-gated chloride channels (Demain, 2000).

2.3.2 Antimicrobial compounds

Nodulisporium spp. fungal endophytes isolated from the endangered plant *Juniperus cedrus* (Canary Island Juniper, a gymnosperm) yielded nodulosporins A–C which exhibited antifungal activity against *Microbotryum violaceum* (Dai *et al*., 2006). Interestingly, the same endophyte was isolated from an Angiosperm shrub, *Erica arborea*, from the Canary Island of Gomera from which related compounds were isolated called nodulisporins D–F (Dai *et al*., 2009). Nodulisporins D–F showed antibacterial activity against *Bacillus megaterium*, antifungal activity against *M. violaceum* (anther smut fungus), and anti-algal activity against *Chlorella fusca.* Several non-viridin-related metabolites, such as hinnuliquinone and the hinnulins, were isolated from cultures of the fungus *Nodulisporium hinnuleum* Smith (ATCC 36102) (Schlingmann *et al*., 2011). Hinnulins were further classified to be members of the 1,2'-binaphthyl class; nodulisporin A and hypoxylone. Compounds of this class have been reported to display herbicidal, antifungal or antibacterial activity (Dai *et al*., 2006) which is probably linked to the propensity of naphthalenediol containing compounds to be sensitive towards oxygen forming radical containing species. *Nodulisporium* sp., an endophytic fungus, isolated from a medicinal plant, *Nothapodytes foetida*, was found to produce antifungal metabolites against *Alternaria alternata* and *Colletotrichum gleosporoides*. The fungal metabolites also showed appreciable growth inhibition mainly active against disease causing Gram positive bacteria (Rehman *et al*., 2011).

2.3.3 Antitumor and anticancer compounds

The anticancer and antifungal drug paclitaxel (Taxol) was reported to be produced by 20 different endophyte species inhabiting different plant species (Zhou *et al*., 2010b). *Nodulisporium sylviforme*, an endophytic fungus of *Taxus cuspidata* Sieb. and Zucc., was found to produce taxol, a functional taxane diterpene amide with anti-tumor activities (Zhou *et al*., 2001). The Endophytic fungus *Nodulisporium* sp. A4 of

Aquilaria sinensis was found to produce a new isofuranonaphthalenone and benzopyrans which showed cytotoxic activities against the NCI-H460 and SF-268 tumor cell lines (Wu *et al*., 2010).

2.3.4 Volatile antimicrobial compounds

Production of volatile antimicrobial compounds by endophytic *Nodulisporium* in cultures has been reported in recent years. *Nodulisporium* sp., an endophyte of *Myroxylon balsamum* in the upper Napo region of the Ecuadorian Amazon (Mends *et al*., 2012), was found to produce volatile organic compounds (VOCs) that have biological potential. Under microaerophilic growth environments, the organism produces 1-methyl-1, 4-cyclohexadiene, 1-4 pentadiene and 1-methyl-4-cyclohexene. The VOCs of *Nodulisporium* sp. were active against a number of pathogens causing death to both *Aspergillus fumigatus* and *Rhizoctonia solani* and severe growth inhibition produced in *Phytophthora cinnamomi* and *Sclerotinia sclerotiorum*. Similarly, the VOCs produced by *Nodulisporium* sp., an endophyte of *Thelypteris angustifolia* (Broadleaf Leaf Maiden Fern) in a rainforest region of Central America, were selectively active against plant pathogens, *Phytophthora palmivora*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Ul-Hassan *et al*., 2012). The most abundant identified VOC was 1,8 cineole. The VOCs produced by *Nodulisporium* sp. CF016, an endophytic fungus of Lauraceae trees collected from regions of Jeju Island, Korea, inhibited and killed a wide range of plant pathogens including *Pythium ultimum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora capsici*, *Sclerotinia sclerotiorum*, *Colletotrichum coccodes*, *Magnaporthe oryzae*, *Alternaria panax*, *Botrytis cinerea* and *Penicillium expansum* (Myung Soo Park *et al*., 2010). Mycofumigation, the use of antimicrobial volatiles produced by fungi for the control of other microorganisms, with wheat bran-rice hull cultures of *Nodulisporium* sp. CF016 showed *in vivo* antifungal activity against gray mold caused by *B. cinerea* and blue mold caused by *P. expansum* of apple. The most abundant volatile compound produced by *Nodulisporium* sp. CF016 was β-elemene followed by 1-methyl-1,4-cyclohexadiene, β-selinene and α-selinene. The endophytic fungus, *Nodulisporium* sp. CMU-UPE34, isolated from *Lagerstroemia loudoni* produced antifungal volatile compounds, primarily composed of alcohols, acids, esters and monoterpene (Suwannarach *et al*., 2013). *In vivo* mycofumigation with jasmine rice grain cultures of *Nodulisporium* spp. CMU-UPE34 controlled green mold decay on *Citrus limon* caused by *Penicillium digitatum*, blue mold decay of *Citrus aurantifolia* and *Citrus reticulata* caused by *P. expansum*.

2.4 The influence of culture medium components on production of fungal secondary metabolites

Under natural environmental conditions, or in culture, the growing fungi utilize the surrounding nutrients as energy sources to produce macromolecules such as proteins, lipids, and nucleic acids for continued growth and biomass production allowing them to proliferate and colonize new regions to obtain more nutrients. Primary metabolites are formed during the active growth of the fungus. When nutrients depletion occurs in a particular environment, or in culture, growth of the fungus slows down while parts of the mycelium switch from primary metabolism to use different biochemical pathways. This alteration in metabolism prevents the fungus from poisoning itself and maintains the biochemical machinery of the cells. Primary metabolites and intermediate compounds accumulated in the fungus are converted to secondary metabolites. Secondary products are not normally produced during active growth and are not essential for vegetative proliferation (Isaac, 1997).

The biosynthesis of fungal secondary products is directly related to the components in culture medium and culture conditions (Elias *et al*., 2006). It usually occurs during the stationary phase (idiophase), and after fungal growth (tropophase). The components in culture medium, e.g., carbon sources, nitrogen sources play significant roles on growth and production of secondary metabolites. However, carbon and nitrogen sources which are favorable for growth may not be beneficial to secondary metabolites production.

2.4.1. Carbon

Carbon sources are mainly used for all growth and product formation. However, the production of secondary metabolite usually occurs when rapidly utilized carbon sources in the media are exhausted; that is, the presence of some carbon sources would repress the formation of secondary metabolite. The most well-known case is the repression of penicillin and cephalosporin production by glucose. In penicillin C formation by *Penicillium chrysogenum*, glucose represses tripeptide formation from L-α-aminoadipic acid, L-valine and L-cysteine (Martin and Aharonowitz, 1983). Formation of cephalosporin C by *Cephalosporium acremonium* is often delayed until the rapidly utilized sugar (glucose) has been consumed and the slowly utilizable sugar (sucrose) begins to be uptaken. To compare individual carbon sources, it was found that readily utilizable carbon sources (i.e. glucose, glycerol, maltose) yield less antibiotic than do slowly utilized sugars (sucrose, galactose). When different concentrations of glucose are employed, the best antibiotic production occurs at the lowest glucose concentration (Behmer and Demain, 1983). The *Cephalosporium* fermentation yields two major products, penicillin N and cephalosporin C. Penicillin N, an intermediate in the formation of cephalosporin C, accumulates extracellularly because the enzyme converting it to desacetoxycephalosporin C is a rate-limiting labile enzyme requiring continuous resynthesis throughout the fermentation. Desacetoxycephalosporin C synthetase (expandase), the enzyme of the pathway, is most susceptible to carbon source repression. It does not appear in the mycelium until glucose is completely consumed and growth has ceased. On the other hand, an earlier enzyme, iso-penicillin N synthetase (cyclase) appears during trophophase as does the extracellular intermediate, penicillin N.

Carbon source control also occurs in mycotoxin producers. Glucose decreases alkaloid production by *Clavicep* (Floss, 1976); polyols and organic acids are the preferred carbon and energy sources. When the enniatin fermentation by *Fusarium sathucinurn* is carried out with glucose (which is rapidly utilized), production occurs only after growth. If the slowly metabolized lactose is chosen as the carbon source, production accompanies fungal growth (Audhya and Russell, 1975). Likewise, *Penicillium cyclopium* does not produce benzodiazapine alkaloids if grown in glucose containing medium. If the glucose is replaced by sorbitol and mannitol, alkaloids are produced (Schroder *et al*., 1978). If alkaloid-producing cells are subjected to glucose addition, there is an immediate inhibition of alkaloid synthesis by over 50%, suggesting that both carbon source repression and inhibition are operative in this organism.

Carbon sources play significant roles on growth and production of secondary metabolite by endophytic fungi. For example, carbon sources affect growth and production of red pigments by endophytic fungus *Paecilomyces sinclairii*. The specific growth rate of *P. sinclairii* in sucrose containing medium was higher than that in starch containing medium, whereas the specific production rate of red pigments was more favorable in starch containing medium (Cho *et al*., 2002). Similarly, carbon sources affect growth and production of secondary metabolite displaying antimicrobial activity by endophytic fungus *Hypocrea* sp. NSF-08. Glucose, starch, lactose, mannitol, sucrose, glycerol, galactose, fructose, maltose, ribose and xylose were supplemented separately into the basal medium. A higher amount of mycelial dry weight was detected in glucose supplemented medium, while glycerol is the best carbon source maximizing the production of antimicrobial agent among other carbon sources (Gogoi *et al*., 2007).

2.4.2 Nitrogen

The type of nitrogen sources is another key factor affecting cell growth and product formation. In general, the biosynthesis of fungal secondary metabolites is limited by nitrogen sources favoring cell growth, such as ammonium salts and certain types of amino acids.

Ammonia (or some other readily utilizable nitrogen source) represses enzymes involved in the metabolism of alternative nitrogen sources such as nitrite reductase, nitrate reductase, glutamate dehydrogenase, arginase, extracellular protease and acetamidase. However, other nitrogen sources, such as proline and urea, support cell growth poorly whilst stimulate the production of secondary metabolites satisfactorily.

Secondary metabolite production is negatively affected by NH_4^+. For example, production of acetate-derived phenolic idiolites such as trihydroxytoluene by *Aspergillus fumigatus* is under nitrogen source control (Ward and Packter, 1974). The production of these polyketides starts, in batch culture, only when nitrogen is exhausted from the medium. Addition of ammonia completely inhibits their production without affecting growth. Bikaverin formation by *Gibberella fujikuroi* is also under nitrogen regulation (Bu'Lock, 1974). When glycine is used as nitrogen source for the fermentation, bikaverin production begins only upon glycine exhaustion. If glycine is added to a resting mycelial suspension, bikaverin production decreased.

Influence of five inorganic nitrogen sources (ammonium sulphate, ammonium nitrate, potassium nitrate, sodium nitrate and ammonium chloride) on the yield of cephalosporin-C (CPC) produced by endophytic *Acremonium chrysogenum* NCIM 1069 were investigated. The ammonium sulphate promotes dry cell weight and the production of CPC better than other inorganic nitrogen sources. Potassium nitrate also led to good yield of CPC followed by ammonium nitrate. The minimum concentration of CPC was attained when ammonium chloride was employed as nitrogen source (Nigam *et al*., 2007).

Culture medium containing leaf extract supplemented with dextrose as carbon source and yeast extract as nitrogen source enhanced the antimicrobial compound production by endophytic *Fusarium* sp. DF2. Culture media containing leaf extract supplemented with other nitrogen sources, i.e., beef extract, $(NH_4)_2SO_4$ and peptone enhanced the biomass production but not the bioactive metabolite production (Gogoi *et al*., 2008).

Lara *et al.* (1982) reported that glutamate could stimulate penicillin production to a much greater degree than glutamine or NH_4^+. Glutamate analogs such as L-glutamic acid, γ-mono-hydroxamate and γ-benzyl-L-glutamate could also stimulate penicillin production due possibly to the fact that glutamate could increase the dipeptide synthetase activity by 3.5 folds.

Mateos *et al*. (1984) found that glutamine at concentration higher than 1 mM inhibited penicillin production of the resting cells of *Penicillium chrysogenum* whereas total inhibition was observed at glutamine concentration of 10 mM.

2.5 One-variable-at-a-time technique versus experimental design

In general, the experimental procedure for optimization is achieved by studying a single factor at any one time. While concentration of one factor is modified to find the optimal response, others are kept at a constant level. The technique is known as the one-variable-at-a-time technique. Clearly, its major disadvantage is that interactions among the factors are not considered so it does not reflect all the potential effects on the process. This means that the supposed ideal level of a factor was determined based on certain levels of other factors. To avoid such problem, Fisher (1935) suggested that all factor-level combinations should be observed by using design of experiments (DOE) which is a factorial experiment. The factorial experiment is able to facilitate the determination of how the factors affect the response through their individual average effects (called the main effects) and through their joint effects (called the interactions).

2.6 Response surface methodology (RSM)

Methodologies that help the experimenter reach the goal of optimum response are referred to as Response Surface Methods. These methods are exclusively used to examine the surface or the relationship between the response and the factors affecting the response. RSM composes of three basic steps which are (i) a factorial or fractional factorial design to screen out irrelevant factors, (ii) after obtaining a regression model for the response, the path of steepest ascent is applied to search for the direction at which response increases the fastest and (iii) a response surface design is employed to obtain the empirical model representing correlation between response of interest and process variables and; thus, the optimum response.

2.6.1 Screening experimental designs

Screening design is an experimental design whose purpose is to distinguish significant factors from non-significant factors as efficiency as possible. Several experimental designs can be used for screening the important factors such as Placket-Burman design, two levels full factorial design and fractional factorial design.

2.6.1.1 Plackett-Burman design

Placket-Burman design is in a class of resolution III, two-level factorial experimental design that allows investigation of many factors simultaneously and inexpensively. These designs are useful to identify the most important variables early in the experimentation phase. They are generally used with eight or more factors. In Plackett-Burman design, main effects have a complicated confounding relationship with two-factor interactions. Therefore, these designs can be used to study main effects assuming that two-way interactions are negligible.

2.6.1.2 Full factorial designs

Full factorial designs are designs in which responses at all combinations of the factor levels are measured. These designs are very useful for preliminary studies or in the initial steps of an optimization. If there are *k* factors, each at 2 levels, full factorial designs require 2^k runs. These screening methods need a large number of runs; thus, are not very efficient when many factors are involved (Box *et al*., 1978).

2.6.1.3 Fractional factorial designs

Fractional factorial designs are designs in which only a selected subset or fraction of the runs in the full factorial design are performed. They are good choices when resources are limited or the numbers of factors in the designs are large because they use fewer runs than the full factorial designs. Fractional factorial designs contain 2^{k-p} runs where *k* is the number of factors and *p* is the number of generators with which assignment as effects or interactions are confounded (Montgomery, 2001). Alias structure may be found by multiplying each of effect columns by the defining relation. For example, half fractional factorial design containing 2^{k-1} runs with 3 factors (2^{3-1} or 2^2 design), A and B and interaction C is written, and these are used to define the levels of variable C, thus C = AB. The AB is called the generator of C. The generator is assigned as the letter I, which is I = ABC called the defining relation. Defining relation is the key to the confounding pattern. For example, A and BC are alias, B and AC are alias, C and AB are alias. Confounding of BC with A indicates that an estimate effect for A includes any effect due to BC. When factor B is confounded with AC indicates that an estimate effect for B includes any effect due to AC.

The 2^{3-1} design is a resolution III design (2^{3-1}_{III}) (Table 2.2). Resolution describes the extent to which effects in a fractional factorial design are aliased to one another. When a fractional factorial design is run, one or more of the effects are confounded, meaning they cannot be estimated separately from one another. In general, a fractional factorial design with the highest possible resolution is required (Montgomery, 2001). Resolution III, IV, and V designs are the most common:

Resolution III designs are those for which no individual factor (main effect) is aliased with another individual factor, but for which at least one factor is aliased with 2-factor interaction.

Resolution IV designs are those for which no individual factor (main effect) is aliased with another individual factor or with 2-factor interaction, but at least one 2-factor interaction is aliased with another 2-factor interaction, and at least one individual factor is aliased with a 3-factor interaction

Resolution V designs are those for which no individual factor (main effect) is aliased with another individual factor or with 2-factor interaction, but 2-factor interaction is aliased with another 3-factor interaction, and main effect is aliased with 4-factor interaction.

Table 2.2 Two-level Fractional Factorial Designs with different resolution

		# of variables/factors								
		3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
# of test runs	**4**	2^{3-1}_{III}								
	8	2^{3}	2^{4-1}_{IV}	2^{5-2}_{III}	2^{6-3}_{III}	2^{7-4}_{III}				
	16		2^{4}	2^{5-1}_{V}	2^{6-2}_{IV}	2^{7-3}_{IV}	2^{8-4}_{IV}	2^{9-5}_{III}	2^{10-6}_{III}	2^{11-7}_{III}
	32			2^{5}	2^{6-1}_{VI}	2^{7-2}_{IV}	2^{8-3}_{IV}	2^{9-4}_{IV}	2^{10-5}_{IV}	2^{11-6}_{IV}
	64				2^{6}	2^{7-1}_{VII}	2^{8-2}_{V}	2^{9-3}_{IV}	2^{10-4}_{IV}	2^{11-5}_{IV}
	128					2^{7}	2^{8-1}_{VIII}	2^{9-2}_{VI}	2^{10-3}_{V}	2^{11-4}_{V}

With the application of screening experimental design for medium optimization, fractional factorial design (FFD) has been widely used for improvement of the fungal metabolites production. Hao *et al.* (2006) studied the effects of the medium components on cellulose production by the recombinant *T. reesei* WX-112 using FFD. The authors found that avicel and soybean cake flour were significant. Macedo *et al*. (2007) adopted FFD to investigate the medium composition for MTGase production by Brazilian soil *Streptomyces* sp. and found that the soybean flour, peptone, KH_2PO_4 and $MgSO_4.7H_2O$ had a significant effect on MTGase production.

2.6.2 Steepest ascent method

The method of steepest ascent is a procedure for moving sequentially along the path of steepest ascent to the region of the optimum, that is, in the direction of the maximum increase in the response. The path of steepest ascent is conducted in reaching for the proper direction for variable manipulation either increasing or decreasing the concentration according to the sign of the main effects obtained earlier in screening design. Based on the results obtained from the FFD, the fitted first-order model is given as:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i \quad (1)$$

Where Y is the predicted response, β_0 is the constant coefficient, β_i is the linear coefficient, and X_i is the coded independent variable. The direction of steepest ascent is the direction in which Y increased most rapidly. The zero level of selected variables in the FFD will be identified as the base point of the steepest ascent path. The steps along the path were determined by the estimated coefficient ratio from Eq. 1, together with the practical experience. Once the path of steepest ascent no longer led to an increase in response, the procedure should be discontinued in favor of a more elaborate experiment. Thus, the steepest ascent method allowed coming closer to the optimal level and locating a new experimental region (Jia *et al*., 2008).

The path of steepest ascent has been successfully applied to achieve appropriate range for optimization. Hao *et al*. (2006) investigated the optimization of the medium for the production of cellulase by the mutant *Trichoderma reesei* WX-112 and found that the optimal point lied outside the experimental design space. The method of steepest ascent was applied. As a result, a path of steepest ascent was adopted leading to the condition near the optimum concentration of 37.5 g/L Avicel and 25.6 g/L soybean cake flour.

2.6.3 Optimization experimental designs (Box *et al*., 1978)

The researchers are often interested in a response of process, which is influenced by several factors, and the aim is the simultaneous optimization of several factors leading to the best performance of a particular system. The Response Surface Methodology (RSM) can be defined as a group of statistical and technical tools used to study the relationship between a response of interest and several input variables, namely, empirical model. RSM is often used to refine models after important factors have been determined using screening experimental designs. The model constructed describes the behavior of a group of data with a view to make statistical predictions.

For example, if the response of a process could be expressed as a function of several process variables as:

$$Y = f(x_1, x_2, \ldots, x_k) + \varepsilon \tag{2}$$

Where Y is observed response, x_i is variable i ($i = 1, 2, \ldots, k$) and ε is random error. The expected response is given by:

$$E(Y) = \hat{Y} = f(x_1, x_2, \ldots, x_k) \tag{3}$$

The response represented by $\hat{Y}$ is called a response surface. If the response is moderately nonlinear, a quadratic model (2nd degree mathematical model) may be appropriate:

$$Y = \beta_0 + \sum_i \beta_i x_i + \sum_i \sum_j \beta_{ij} x_{ij} + \sum \beta_{ii} x_i^2 \tag{4}$$

where Y is the measured response, β_0 *is* the intercept term, β_i , β_{ij}, and β_{ii} are the measures of the effects of variables x_i, x_{ij} , and x_i^2, respectively.

The most commonly employed second-order designs for the RSM include the Box-Behnken design (BBD), the Central Composite Design (CCD) and Doehlert's Design. These differ by the location of the experimental points in the studied region, the number of factor levels kept, the number of experiments and the blocks. Central Composite Designs (CCD) are one of most common design that can fit a full quadratic model. They are often used when the design plan calls for sequential experimentation because these designs can incorporate information from a properly planned factorial experiment. The CCD contain an imbedded factorial or fractional factorial matrix with center points and axial point commonly referred to as star points around the center point that allow estimation of curvature as shown in Figure 2.3.

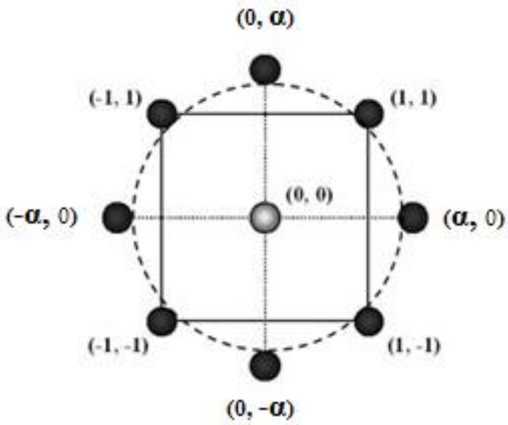

Figure 2.3 Schematic representation of CCD for two factors

If the distance from the center of the design space to a fractional point is ±1 unit for each factor, the distance from the design space to a star point is ±α, where $|\alpha| > 1$. The precise value of α depends on certain properties needed for the design and on the number of factors, α = [number of factorial runs] $^{1/4}$. If the factorial is a full factorial, then α = ± $[2^k]^{1/4}$. For example, if the study contains 2 factors k = 2 factors, subsequently, α = ±1.141 (Figure 2.3).

The central composite design (CCD) has found application in optimization of fungal growth, biochemical production, and biologically active secondary metabolite production. Macedo *et al*. (2007) optimized the medium composition for MTGase production from Brazilian soil *Streptomyces* sp. using CCD. It was found that the ability to produce MTGase before using statistical method is 0.25 U.mL^{-1}. Under the obtained optimized conditions, the model predicted a MTGase activity of 1.37 U.mL^{-1}, closely matched the experimental activity of 1.4 U.mL^{-1}. Jia *et al*. (2008) adopted CCD to optimize nutrient levels for the production of pristinamycins by *Streptomyces pristinaespiralis* CGMCC 0957, together with the steepest ascent method. Under the optimized conditions, the yield of pristinamycins in the shake flask and 5-L bioreactor reached 1.30 and 1.01 g/L, respectively, which are the highest yields reported in literature to date.