17 Bioprocessing of Endophytes for Production of High-Value Biochemicals

Khwajah Mohinudeen, Karthik Devan, and Smita Srivastava

17.1 Introduction

The term endophyte literally translates into 'within a plant' and was initially coined to refer to organisms living inside a plant (Chaichanan et al. 2014). However, it is currently used in the context of mutualistic fungi and bacteria living inside plants. Endophytes have been found in plants belonging to every plant family (Ray et al. 2017; Singh et al. 2017). There are several hypotheses regarding endophyte-plant relationship, and it is believed that plants harbouring endophytes are healthier than their endophyte-free counterparts (Martinez-Klimova et al. 2017). The symbiotic relationship seems beneficial to the endophyte as nutrients for growth are available from the plant. Endophytes promote plant growth by fixing nitrogen, helping in the uptake of mineral nutrients such as phosphorus and iron (Thiry and Cingolani 2002). Endophytes are also said to modulate the levels of phytohormones (Santoyo et al. 2016). Endophytes also defend the plants against pathogens and insects by producing secondary metabolites. Many metabolites isolated from endophytes are found to exhibit antimicrobial (Golinska et al. 2015) and antifungal (Ola et al. 2013) activity. Another important hypothesis is that endophytes compete with pathogens in colonizing plant tissues and therefore help in minimizing damages caused by pathogens. Endophytes have also evolved to overcome plant defences and thrive inside their host plant. Endophytes are of special interest because they have been found to synthesize chemical compounds that are also known to be produced by their host plant, such as taxol and camptothecin (Thiry and Cingolani 2002). Apart from host-identical compounds, several other compounds such as antibiotics and bioactive peptides that are of commercial interest are also produced by endophytes (Castillo et al. 2002; Ezra et al. 2004). Further, the advances in analytical

K. Mohinudeen · K. Devan · S. Srivastava (✉)
Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences Building, Indian Institute of Technology Madras, Chennai, India
e-mail: smita@iitm.ac.in

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techniques, such as gas/liquid chromatography coupled with mass spectrometry and liquid chromatography coupled with nuclear magnetic resonance, have led to recent interest in bioprospecting of endophytes for characterization and identification of known and novel bioactive compounds with relative ease, for prospective commercial applications. In addition the biochemical production from endophyte can be improved through various strategies. Bioprocess condition optimization can help in enhancing the productivity (Singh et al. 2016). Exogenous addition of elicitors helps in stress-induced production of metabolites, and precursor addition helps in driving the biosynthetic pathway towards metabolite formation. Also strain improvements through genetic modifications can help in overcoming their drawback of unsustainability. Diverse methods and developments towards bioprocessing and bioprospecting are discussed below in this chapter.

17.2 Endophytes: Discovery and Terminology

Until the nineteenth century, it was believed that healthy growing plants are sterile and devoid of microbiota as hypothesized by Pasteur (Compant et al. 2012). Endophytes were first described by the German botanist H.F. Link in 1809 (Link 1809), and over the next few years, endophytes were defined in numerous ways. Béchamp referred to microorganisms living in plants as microzymas (Béchamp 1866). A few years later, Galippe reported the occurrence of microorganisms – fungi and bacteria – in the interior parts of vegetable plants (Galippe 1887). The initial assumption that all microbes living inside plant hosts are parasitic in nature was disproved by the Dutch microbiologist Martinus Willem Beijerinck (Beijerinck 1888). His discovery of rhizobium bacteria present in root nodules of leguminous plants, which help in fixing atmospheric nitrogen, was a major breakthrough. Another important discovery was the symbiosis between roots of trees and underground fungi which was reported by Albert Bernhard Frank who coined the term 'mycorrhiza' (Frank 1885). A number of other studies confirmed the occurrence of microbes in plants with reports ranging from parasitic organisms to beneficial ones. Currently, it is a renowned fact that many types of microbial endophytes, including bacteria, fungi, archaea and protists such as algae (Trémouillaux-Guiller et al. 2002) and amoebae (Müller and Döring 2009), utilize plants as their host for living. In the 1990s, endophytes were defined as all organisms residing within plants at some time in their life cycle, colonizing their internal tissues without creating any evident harm to the hosts (Petrini 1991). However, the definition has undergone numerous transitions with time. While some microbes may be living as dormant pathogens in plants and turn out to be pathogenic under particular environments (Kloepper et al. 2013), others may be beneficial and growth promoting to a specific plant species and pathogenic to another plant. Thus, establishing a crystal clear definition for endophytes has been an arduous task. Microbial numbers, genotype of plant and microbes, environmental circumstances and quorum sensing are important factors to be considered while distinguishing between pathogenic and non-pathogenic endophytes. As the word suggests, *endo* (inside) and *phyte* (plant), the term could be

used to refer to only the habitat that all microbes live inside the plant host for a part or all of their lifespan regardless of the function. As of today, endophytes have been identified to be dwelling in every plant family.

17.3 Plant-Endophyte Relationship

The presence of endophytic fungi has been traced back to 400 million-year-old Devonian Rhynie chert deposits from fossil records of plants which lacked a rooting system. Fungi and Peronosporomycetes (organisms similar to fungi) were ubiquitous and spread out widely on the Earth before the first appearance of land plants during the Silurian period of the Palaeozoic era (Taylor and Taylor 1993). Initial land plants lacked proper leaves or roots in them until the Devonian period, during which they developed prominent rooting system and leaves (Beerling et al. 2001; Raven and Edwards 2001). However, even the most ancient preserved land plants, which are deficient of distinct leaves, roots and shoots, had fungal endophytes present in them (Krings et al. 2012). This clearly states that plants have evolved along with fungi and other microbes, which were present on the Earth before them, and plants had adapted to exist on Earth along with the endophytes in them during their period of evolution.

The nature of plant-endophyte interactions ranges from mutualism to pathogenicity depending on numerous biotic and abiotic factors including genotypes of the plant and the microbe, environmental conditions and dynamic interactions within the plant biome. Endophytes promote plant growth by fixing nitrogen, helping in the uptake of mineral nutrients such as phosphorus and iron (Alvin et al. 2014). Endophytes also modulate the levels of phytohormones such as auxin, cytokinin, gibberellin and ethylene in plants (Martinez-Klimova et al. 2017). Endophytic fungi are also known to help the plants in which they reside by assisting them in acclimatization towards various stress factors (heat, salinity, drought, diseases, herbivores, etc.) (Rodriguez et al. 2009). *Curvularia* sp., isolated from *Dichanthelium lanuginosum*, has portrayed improved heat resistance on the host plant. Similarly, *Fusarium culmorum* is found to increase the tolerance against salinity in the host plant *Leymus mollis* (Rodriguez et al. 2009).

Endophytes can remain in plant tissues throughout their lifespan. When the plant parts, like leaves, fall off, they can continue to survive in the fallen leaves of host plants by converting into saprophytes and help in degradation (Korkama-Rajala et al. 2008; Voriskova and Baldrian 2013; Prakash et al. 2015). Endophytes undergo up-regulation of several genes in order to support this conversion to saprophytes (Zuccaro et al. 2011).

Though the mechanism and role of endophytes in plants are still under study, there are various hypothesis proposed on the endophyte's properties (Kusari et al. 2012a). One of those is mosaic theory according to which the endophytes create a chemical environment in the host plant tissue which prevents them from phytophagous and pathogens (Carroll 1991). In another parallel theory, endophytes are addressed as acquired immune systems for the plant in which they reside (Arnold

et al. 2003). An even more topical hypothesis called xenohormesis (Howitz and Sinclair 2008) states that evolutionarily certain microbes might have attained the potential to sense stress-induced signalling molecules from plants and also the competence to synthesize the bioactive compounds, due to selection pressure. However, with time, the heterotrophs might have lost the potential to synthesize the compounds, or the genes responsible for synthesis might have got silenced, and they only retain their sensing ability (Kusari et al. 2012a). Recently, several natural products which were believed to be restricted only in plants are found to be synthesized by microbes and animals. For example, morphine which was earlier reported only from plants (*Papaver somniferum*) was discovered even in mammals (Grobe et al. 2010). Similarly, several metabolites produced by natural plants were reported to be produced by the endophytes as well. In fact, there is also a possibility that some of the metabolites produced by the natural plants are the byproducts of the endophytes residing in the plants (Kusari et al. 2012a).

17.4 Production of High-Value Plant Secondary Metabolites

Plants produce certain bioactive compounds which are not essential for their growth but are defence response towards the environmental stress factors. These compounds are generally termed as 'secondary metabolites' which have various medicinal applications. Secondary metabolites produced by plants include alkaloids, terpenoids, flavonoids, steroids, peptides, quinols, phenols and polyketones, which have several medicinal properties like anticancer, antimicrobial, immunesuppressive, anti-inflammatory and antioxidant (Korkina 2007).

From the statistical point of view, it is clear that plants play a vital role in the worldwide drug market with 25% of the approved drugs being originated from plants, and among the 252 generic drugs acknowledged by the WHO, 11% are plant-based drugs (Dubey et al. 2012). A minimum of 120 plant-based active compounds are in regular practice in most countries (Taylor 2005). Besides, ~47% of the anticancer drugs actively being used worldwide are plant-derived natural products (Newman and Cragg 2007). WHO has reported recently that nearly 60,000 plant species across the world have been estimated to be used for their medicinal properties, leading to 500,000 tons of the plant material being traded annually worldwide with a market value of USD 2.5 billion (Dushenkov 2016). Increased trading has reduced most plant population drastically, with only 1.4% remaining on the Earth's surface (Dushenkov 2016). The tropical rainforests which have the largest diversity of plant species are plunging at swift rate from 14% to a meagre 6% with not even 1% of them being focused towards novel drug discoveries, which eventually may result in several species getting extinct without even studying them for valuable metabolites (Taylor 2005). Hence, there is a severe need to reduce the dependence on the plants for their metabolites by shifting towards alternate and sustainable sources of such metabolites.

Though in vitro plant cell culture techniques are visualized as commercial alternatives for plant secondary metabolite production, they suffer from limitations

including scale-up difficulties, low productivities, contamination risk, need of expensive phytohormones and genomic instability (Howat et al. 2014). Hence, production of secondary metabolites using plant cell cultures on a commercial scale is not much successful except in few cases such as ginseng, shikonin, berberine and taxol (Linden 2006). In case of camptothecin, though there are reports on plant cell culture production, they are not yet commercially successful (Kai et al. 2015). Apart from this, chemical synthesis is also looked forward as potential substitute for such metabolite production. However, commercial trials on total chemical synthesis of complex plant secondary metabolites have mostly resulted in failure, except for a few simple structured compounds like vanillin, whose demands have been widely substituted with synthetic vanillin (Koeller and Wong 2001). Chemical synthesis of compounds like morphine is uneconomical owing to complications in their sterical structure with five chiral centres. Similarly for chemical synthesis of paclitaxel, 40 steps of processing are required which finally results in a low product yield of less than 5% (Holton et al. 1994a, b). Camptothecin when attempted to be synthesized chemically also resulted in a low yield of 14% with losses in many intermediate steps (Yu et al. 2012).

Another method of production is heterologous expression of genes involved in the biosynthesis pathway. In taxol, a number of steps in the pathway are catalysed by cytochrome P450 (cP450) acyltransferases and oxygenases (Howat et al. 2014). Functional expression of these cP450s in microbial systems such as *Escherichia coli* and *Saccharomyces cerevisiae* has been the bottleneck in taxol synthesis using heterologous microbial hosts, as cP450s fold incorrectly and are inserted into the cell membrane in these systems (Howat et al. 2014). Further, expression of taxol biosynthesis genes in a plant host, *Arabidopsis thaliana*, led to growth retardation (Besumbes et al. 2004)**.** Heterologous production using microbial host could not be achieved for metabolites like camptothecin, since the complete biosynthetic pathway has not been elucidated (Kai et al. 2015). This is the case for many other similar metabolites like podophyllotoxin, vincristine and vinblastine.

17.5 Secondary Metabolite Production by Endophytes: An Alternate Route?

Endophytes, which reside in the plants throughout the plants' lifetime, have attracted researchers from around the globe for their potential to produce the same secondary metabolites as that of the host plant. The first such reported endophytic fungus, *Taxomyces andreanae*, producing taxol was isolated from *Taxus brevifolia* during the early 1990s (Stierle et al. 1993). Over the past decade, reports on endophytes producing plant secondary metabolites have increased by more than tenfolds. Certain commercially significant metabolites produced by the endophytes and their host plants are illustrated in Fig. 17.1.

Cultivation of endophytes under in vitro conditions is more economical in comparison to plant cell culture due to low-cost substrate and other nutrient requirements for microbial fermentation. Unlike plant cell cultures, endophytes do not

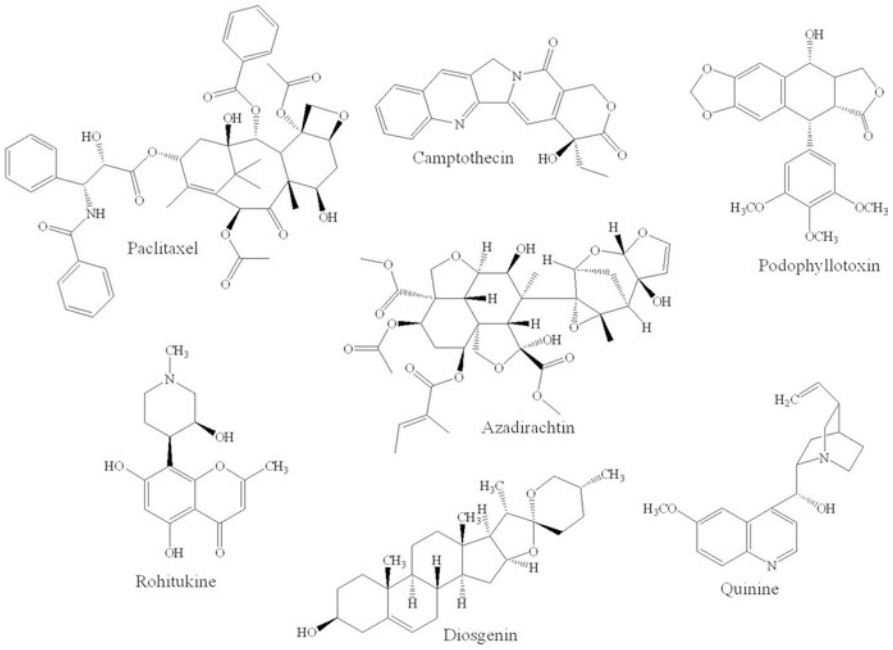

Fig. 17.1 Structures of selected commercially important secondary metabolites produced from plants and also the endophytes isolated from them

have hormonal requirements for growth. Production on industrial scale can be done using waste products such as molasses or whey liquid, which can make the process even more simple and cost-effective (Venugopalan and Srivastava 2015). It is difficult to maintain sterility for longer cultivation period in plant cells due to slow growth rates in comparison to microbes. In case of camptothecin production, plant cells were incubated for a period of 3 weeks (van Hengel et al. 1992; Karwasara and Dixit 2013), whereas endophytes were incubated only for 4 days (Shweta et al. 2010) to 7 days (Puri et al. 2005). Fermentation condition optimization and scale-up process are simpler in case of microbes over plant cell cultivations. Also, implementation of yield improvement techniques such as addition of elicitor and supply of precursor is easily adaptable (Zhao et al. 2010). Endophytic production of metabolites can prevent over-exploitation of the natural plant sources and are also a sustainable source in comparison to plants which vary in their yield depending on developmental stages and seasonal variation (Vance et al. 1994; Liu et al. 1998; Pai et al. 2013).

Though metabolites extracted from endophytes demonstrate a wide range of commercial applications, a major deterrent to commercial exploitation of endophytes has been the widely reported problem of product yield attenuation with subculture (Table 17.1), which can make them a non-sustainable and non-reliable source at large scale. However, it is reported that these disadvantages can be surmounted through optimization of bioprocess parameters and by triggering the

Table 17.1 Attenuation in the metabolite yield by endophytes

Endophytic fungus	Host plant	Metabolite produced	Yield of different generations								References
			1st	2nd	3rd	4th	5th	6th	7th	8th	
Periconia sp. 2026	*Torreya grandifolia*	Taxol	350 ng L^{-1}	325 ng L^{-1}	290 ng L^{-1}	200 ng L^{-1}	118 ng L^{-1}				Li et al. (1998)
Fusarium solani INFU/CA/KF/3	*C. acuminata*	Camptothecin	~6 μg g^{-1}	~5.5 μg g^{-1}	~0.5 μg g^{-1}	~1 μg g^{-1}	~1 μg g^{-1}	~0.5 μg g^{-1}	~0.4 μg g^{-1}		Kusari et al. (2009b)
Phomopsis sp. UAS014	*N. nimmoniana*	Camptothecin	+	21.7 μg g^{-1}	11.4 μg g^{-1}	6.6 μg g^{-1}					Gurudatt et al. (2010)
Aspergillus sp. LY341	*C. acuminata*	Camptothecin	7.93 μg l^{-1}	<LOD							Pu et al. (2013)
Aspergillus sp. LY355	*C. acuminata*	Camptothecin	42.92 μg l^{-1}	4.06 μg l^{-1}	<LOD						
T. atroviride LY357	*C. acuminata*	Camptothecin	197.82 μg l^{-1}	5.33 μg l^{-1}	2.57 μg l^{-1}	2.47 μg l^{-1}	3.69 μg l^{-1}	2.15 μg l^{-1}	1.90 μg l^{-1}	1.83 μg l^{-1}	
F. oxysporum MTCC-11383	*Dysoxylum binectariferum*	Rohitukine	~1.9 μg g^{-1}	~1 μg g^{-1}	~0.8 μg g^{-1}	~0.6 μg g^{-1}					Kumara et al. (2014)
G. fujikuroi MTCC-11382	*Amoora rohituka*	Rohitukine	~1.8 μg g^{-1}	~1.3 μg g^{-1}	~1.1 μg g^{-1}	~0.8 μg g^{-1}					

cryptic pathways for metabolite synthesis in the endophytes (Venugopalan and Srivastava 2015). Table 17.1 lists some of the reports, which show product yield attenuation with subculture in the axenic cultures of endophytes.

17.5.1 Antimicrobial and Anticancer Compounds Produced by Endophytes

17.5.1.1 Antimicrobials

In the past century, antimicrobial compounds such as antibiotics have proven indispensable in combating microbial infections not only in humans but also in other areas such as agriculture. However, this has also led to the evolution of antibiotic-resistant strains. It was estimated that nearly 25,000 people died in Europe in 2009 due to infections caused by multiple drug-resistant bacteria (Freire-Moran et al. 2011). Hence, the need of the hour is the development of novel antimicrobial compounds to combat multidrug-resistant bacteria.

Endophytes produce a wide range of antimicrobial compounds, presumably to compete with the other microorganisms residing in the plant tissues and prevent their colonization. Therefore, bioprospecting of endophytes can be a promising alternative for discovery of novel antimicrobial compounds. The antibiotic compounds reported from endophytic fungi majorly belong to the phylum *Ascomycota* and that from the endophytic bacteria are from the phylum *Actinobacteria* (Martinez-Klimova et al. 2017). Antibiotic-producing endophytes have been isolated from a diverse variety of plants, globally (Martinez-Klimova et al. 2017). Methanol, ethyl acetate and hexane extracts from *Colletotrichum gloeosporioides*, an endophyte isolated from *Vitex negundo* by Arivudainambi et al. (2011), showed inhibitory activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Further, the extracts showed a synergistic effect when used with common antibiotics such as penicillin and methicillin, opening up the avenues for new means of combating microbial infections. Rani et al. (2017) isolated 20 different fungal endophytes from the medicinal plant *Calotropis procera*, out of which 7 showed antimicrobial activity against various species of bacteria belonging to the genus *Salmonella*. There has been an increase in the number of studies that show endophytes from medicinal plants being a source of antimicrobial metabolites (Dar et al. 2017). Table 17.2 lists few examples of antimicrobial compounds identified from isolated endophytes in literature.

17.5.1.2 Anticancer Agents

The scientific pursuit of using plant-derived metabolites as anticancer agents started with vinblastine and vincristine in 1950 (Chandra 2012). Thereafter, several compounds from plants have been used for the production of clinically useful anticancer drugs. Major compounds on that list include taxol, camptothecin, vinblastine, vincristine and podophyllotoxin.

Table 17.2 Bioactive metabolites produced by some isolated endophytes

Metabolite	Bioactivity	Endophyte	Host plant	Yield	References
Azadirachtin	Biopesticide	*Eupenicillium parvum*	*Azadirachta indica*	0.4 µg/100 gm 43 µg/L	Kusari et al. (2012b)
Camptothecin	Anticancer	*Entrophospora infrequens*	*Nothapodytes foetida*	49.6 µg/gm	Amna et al. (2006)
		Fusarium solani (MTCC 9667)	*Apodytes dimidiata*	0.37 µg/gm	Shweta et al. (2010)
		Fusarium solani (MTCC 9668)	*Apodytes dimidiata*	0.53 µg/gm	
		Nodulisporium sp.	*Nothapodytes foetida*	5.5 µg/gm	Rehman et al. (2008)
Deoxypodophyllotoxin	Anticancer	*Aspergillus fumigatus* (INFU/Jc/KF/6)	*Juniperus communis*	3 µg/L	Kusari et al. (2009a)
Diosgenin	Progesterone precursor, cholesterol lowering activity	*Cephalosporium* sp. (84)	*Paris polyphylla* var. *yunnanensis*	(+)	Zhou et al. (2004) and Xiao-dong et al. (2007)
Huperzine A	Neurodegenerative disease treatment	*Acremonium* sp. (2F09P03B)	*Huperzia serrata*	(+)	Li et al. (2007)
		Blastomyces sp. (HA15)	*Phlegmariurus cryptomerianus*	(+)	Ju et al. (2009)
		Botrytis sp. (HA23)	*Phlegmariurus cryptomerianus*	(+)	
		Penicillium chrysogenum (SHB)	*Lycopodium serratum*	(+)	Zhou et al. (2009)
Hypericin	Antidepressant, anti-inflammatory, antimicrobial, antioxidant, antiviral	*Chaetomium globosum* (INFU/Hp/KF/34B)	*Hypericum perforatum*	(+)	Kusari et al. (2008)
Podophyllotoxin	Anticancer	*Alternaria neesex* (Ty)	*Sinopodophyllum hexandrum*	2.4 µg/L	Cao et al. (2007)
		Fusarium oxysporum (JRE1)	*Juniperus communis*	28 µg/g	Kour et al. (2008)
		Phialocephala fortinii (PPE5, PPE7)	*Sinopodophyllum peltatum*	0.5–189 µg/L	Eyberger et al. (2006)
		Trametes hirsuta	*Sinopodophyllum hexandrum*	30 µg/gm	Puri et al. (2006)

(continued)

Table 17.2 (continued)

Metabolite	Bioactivity	Endophyte	Host plant	Yield	References
Quinine	Antimalarial	*Diaporthe* sp.	*Cinchona ledgeriana*	30 µg/l	Maehara et al. (2011)
		Arthrinium sp.	*Cinchona ledgeriana*	50 µg/l	Maehara et al. (2013)
		F. solani	*Cinchona calisaya*	0.9 mg/l	Hidayat et al. (2015)
		F. oxysporum		0.9 mg/l	
		F. incarnatum		0.8 mg/l	
Paclitaxel	Anticancer, antiviral	*Alternaria alternate* (TPF6)	*Taxus chinensis* var. *mairei*	84.5 µg/l	Tian et al. (2005)
		Aspergillus fumigatus(EPTP-1)	*Podocarpus* sp.	557.8 µg/l	Sun et al. (2008)
		Cladosporium cladosporioides (MD2)	*Taxus media*	800 µg/l	Zhang et al. (2009)
		Fusarium solani (Tax-3)	*Taxus chinensis*	163.35 µg/l	Deng et al. (2009)
		Metarhizium anisopliae (H-27)	*Taxus chinensis*	846.1 µg/l	Liu et al. (2009)
		Pestalotiopsis neglecta (BSL045)	*Taxus cuspidata*	375 µg/l	Kumaran et al. (2010)
		Pestalotiopsis versicolor (BSL038)	*Taxus cuspidata*	478 µg/l	
		Tubercularia sp.(TF5)	*Taxus chinensis var. mairei*	185.4 µg/l	Wang et al. (2000)
Rohitukine	Anti-inflammatory, immuno-modulatory, anticancer	*Fusarium proliferatum* (MTCC 9690)	*Dysoxylum binectariferum*	1.9 µg/gm	Kumara et al. (2014)
Toosendanin	anti-botulismic agent; agricultural insecticide	Unidentified (O-L-5, O-SC II-4, O-RC-3)	*Melia azedarach*	(+)	Wang et al. (2007a)
Vincamine	Vasodilator, cerebral stimulant	Unidentified (Vm-J2)	*Vinca minor*	0.1 mg/L	Yin and Sun (2011)
Vinblastine	Anticancer	*Alternaria* sp. (97CG1)	*Catharanthus roseus*	(+)	Guo et al. (1998)
Vincristine	Anticancer	*Fusarium oxysporum* (97CG3)	*Catharanthus roseus*	(+)	Zhang et al. (2000)
		Unidentified (97CG1)	*Catharanthus roseus*	0.205 µg/L	Yang et al. (2004)

Taxol

Taxol was initially isolated from the bark of yew tree, *Taxus brevifolia* (Wani et al. 1971). Several other species from the genus *Taxus* were later reported to produce taxol. It is widely used for the treatment of ovarian, breast, lung, head, neck, renal, prostate, colon, cervix, gastric and pancreatic cancers (Zhou et al. 2010). The taxol-producing trees are not abundantly found in nature, and they also grow very slowly. The compound is found only in trace amounts (Zhou et al. 2010), as low as 0.01% dry weight of the bark (Zhou et al. 2010). Increasing demand for the drug has led to indiscriminate exploitation of taxol-producing trees, and it has become important to seek alternative, sustainable methods of producing taxol.

After the discovery of first taxol-producing endophyte in 1993, several similar endophytes with varying yields have been isolated. Interestingly taxol-producing endophytes have been isolated not only from taxol-producing plants but also from other plants such as chilli (*Capsicum annuum*) (Kumaran et al. 2011) and hazel (*Corylus avellana*) (Yang et al. 2014). Kumaran et al. (2011) reported a yield of 687 μg/L from the endophyte *Colletotrichum capsici* they isolated from the chilli plant, which is higher than what is usually seen in the case of endophytes isolated from taxol-producing plant species. Yang et al. (2014) further sequenced the entire genome of the taxol-producing endophyte *Penicillium aurantiogriseum* NRRL 62431 which they had isolated from the hazel plant and detected candidate gene sequences that could be involved in taxol biosynthesis. By comparison of these genes with taxol biosynthesis genes from plants, it seems unlikely that the genes were horizontally transferred to this fungus from a plant host. Apart from isolation of taxol-producing endophytes, several bioprocess strategies have also been applied to achieve yield enhancements, and they are discussed later in the chapter. At this moment, one may say that taxol is the most popularly sought after product with research in bioprospecting and bioprocessing of endophytes.

Camptothecin

Camptothecin is a pentacyclic quinoline alkaloid used as a potent anticancer agent. Camptothecin and its derivatives find applications in the treatment of lung, breast, cervical and uterine cancers (Chandra 2012). Wall et al. (1966) first isolated camptothecin from the wood of the tree, *Camptotheca acuminata*. Other plants reported to contain camptothecin include *Nothapodytes nimmoniana*, *Ophiorrhiza*, *Ervatamia heyneana* and *Merrilliodendron megacarpum* (Chandra 2012). The scenario in camptothecin production is very similar to that of taxol, with the yield from natural sources being very low and the increasing demand leading to exploitation of the natural sources of the compound. The first report of a camptothecin-producing endophyte was by Puri et al. (2005). The fungus was isolated from the host plant, *Nothapodytes nimmoniana* and identified as *Entrophospora infrequens*. Reports on isolation of camptothecin-producing endophytes have been tabulated in Table 17.2. The major bottleneck in scaling up camptothecin production using endophytes is yield attenuation. In a study by Pu et al. (2013) on a camptothecin-producing endophyte *Trichoderma atroviride* LY357, yield attenuation was observed on repeated subculturing. The yield decreased but however was detectable even after eight

generations of subculturing and increased by 50-fold when optimization strategies were applied. This suggests that endophytes lose their biosynthetic capability in the absence of stimulus and regain their capability when appropriate stimuli are applied externally.

Even bacterial endophytes have been reported to produce camptothecin with anticancer activity (Shweta et al. 2013; Soujanya et al. 2017). In the latter case, the production of camptothecin by an endophytic strain of *Bacillus subtilis* attenuated on subculturing and completely ceased when it was cured of a plasmid it harboured. It is therefore possible that the plasmid contained key genes involved in camptothecin biosynthesis. Apart from camptothecin, endophytes have been shown to produce even derivatives of camptothecin such as 10-hydroxycamptothecin (Liu et al. 2010; Shweta et al. 2010) and 9-methoxycamptothecin (Shweta et al. 2013).

Podophyllotoxin

Podophyllotoxin is a pharmaceutically active lignan compound, reported to occur in both gymnosperm and angiosperm plants belonging to the families Cupressaceae, Berberidaceae, Polygalaceae, Lamiaceae and Linaceae (Chandra 2012). *Podophyllum hexandrum* is now declared as 'critically endangered', and also agricultural production of podophyllotoxin by cultivation of *Podophyllum* plants has been unsuccessful due to unsuitable climatic conditions (Chandra 2012). The first report of a podophyllotoxin-producing endophyte was by Yang et al. (2003). Subsequently, several fungal endophytes belonging to the genera *Alternaria*, *Trametes*, *Phialocephala*, *Fusarium* and *Aspergillus* have been reported to produce podophyllotoxin. Yields as high as 189 μg/L have been reported for podophyllotoxin from endophytes (with the endophyte *Phialocephala fortinii* isolated from *Podophyllum peltatum*) (Eyberger et al. 2006). In another study Nadeem et al. (2012) isolated a strain of *Fusarium solani* from the roots of *Podophyllum hexandrum* that could yield 29.0 μg/g of podophyllotoxin. The maximum yield was obtained on the 8th day of cultivation, and application of bioprocess optimization strategies could increase the yield further. A few more examples of such species, along with their host plants, yield and reference, have been listed in Table 17.2.

Vincristine and Vinblastine

Vincristine and vinblastine are alkaloids obtained from the plant *Catharanthus roseus*, commonly known as the Madagascar periwinkle. They can lower the number of white blood cells (Chandra 2012) and are hence used in the treatment of lymphoma and leukaemia. Though *Catharanthus roseus* is not endangered and can be easily cultivated in agricultural fields, the yield of vincristine and vinblastine from these plants is very low. To produce 1 g of vincristine, about 500 kg of *C. roseus* leaves are required (Yue et al. 2016). As the worldwide demand for vincristine and vinblastine is largely met by agricultural cultivation of *Catharanthus roseus,* not much of research has been focused on endophytes for producing these alkaloids. However, endophytes may offer a significantly cost-effective alternative in the future. The first report on a vinblastine-producing endophyte was by Guo et al. (1998) and that on vincristine was by Zhang et al. (2000). Palem et al. (2015) isolated 22 fungal

endophytes with the goal of discovering endophytes that produce vincristine and vinblastine. They tested them for anti-proliferative activity using HeLa cells. They also screened the fungi for the presence of the tryptophan decarboxylase (TDC) gene, which is a key gene involved in the synthesis of terpene indole alkaloids, the class to which vincristine and vinblastine belong to. *Talaromyces radicus* showed the highest anti-proliferative activity and was the only isolated species which contained the TDC gene. On further analysis, it was found that this species indeed could produce vincristine (670 μg/L) and vinblastine (70 μg/L). Endophytes that are known to produce vinblastine and vincristine are listed in Table 17.2.

Apart from antibiotics and anticancer agents, compounds isolated from endophytes show potential use as antidiabetics (Uzor et al. 2017), anti-inflammatory (Gao et al. 2008), antiviral (Zhao et al. 2010), antidepressants (Zhao et al. 2010) and antioxidants (Zhao et al. 2010).

17.6 Bioprospecting of Endophytes for Identification of Useful Metabolites

17.6.1 Isolation of Endophytes from the Host Plant

Isolation of endophytes is the initial process towards bioprospecting endophytes for metabolite production. A host plant contains a wide range of endophytes distributed throughout the plant. Hence, the endophytes isolated from any natural plant may vary with the type of plant tissue selected, the environmental factors and the developmental stage of the plant (Fisher et al. 1993; Collado et al. 1999).

Followed by the selection of explants, surface sterilization of the selected explants is carried out to get rid of various epiphytes and surface contaminants. It is necessary to carry out the surface sterilization on fresh explants so that the microorganisms inside the plant tissue are viable. In case if it is impossible to perform surface sterilization immediately, then it is mandatory to refrigerate the explants to restrain the microorganisms from death (Golinska et al. 2015). Surface sterilization process is a critical step as it decides the fate of the isolated microorganism, if it is an endophyte or an epiphyte (Verma et al. 2009). Examples of some of the surface sterilization protocols from literature are compiled and listed in the Table 17.3. Exposing the tissue to highly concentrated sterilizing reagents or longer exposure time to the reagents might result in destruction of the microorganisms residing within the tissues, and hence additional care should be taken while performing this step (Golinska et al. 2015). Post surface sterilization treatment, adequate washing of the explant is done with sterile distilled water to prevent any harmful effects caused by residual amount of surface-sterilizing agents. Further, to check the effectiveness of surface sterilization, water used for final wash of the explants is streaked onto a suitable agar plate and observed for any visible growth of microorganisms. Alternatively, the imprint of the explants can be taken on a suitable agar plate and observed for any visible growth of microorganisms. After surface sterilization, the explants are wounded and placed on suitable agar plates (Golinska et al. 2015).

Table 17.3 Various surface sterilization protocols for isolation of plant endophytes

Explant	Surface sterilization	Time of exposure	Excision	Medium	Brief description	References
Leaf and outer stem bark	Distilled water	Thorough wash	Random (0.5 cm^2)	Initially aqueous agar	When hyphae emerged from cut region, single hyphal tips were isolated and subcultured on rich medium PDA (28+/−2 °C). Brought to pure by serial subculturing	Shweta et al. (2010)
	70% ethanol	1 min		Later with rich medium (PDA)		
	5% NaOCl	5 min				
	Sterile water	3 times				
Leaves (~2 gm/tree)	Distilled water	Thorough wash	6 fragments per leaf (6 mm dia)	1.5% MEA	48-well plates were used. 1 ml of MEA in each well. One fragment placed on each well. Incubated at daylight at 20–22 °C for 4 weeks. The leaf fragments, grouped according to their macroscopic appearance (e.g. senescence, discolouration) and morphological-anatomical characters of their fungal colonies, such as sporulation, colony colour and diameter	Unterseher and Schnittler (2009)
	70% ethanol	2 min	Base with middle vein, centre left of middle vein, centre right of middle vein, margin right, margin left, tip			
	1% NaOCl	5 min				
	70% ethanol	1 min				
	Sterile water					
Yew bark (0.5 × 0.5 × 0.5 cm)	70% ethanol, sterile water		Outer bark removed	Small pieces of inner bark placed on PDA	Incubated at 25 °C for at least 2 weeks. Pure cultures transferred to another agar plate by the hyphal tip method. Identification methods were based on the morphology of the fungal culture, the mechanism of spore production and the characteristics of the spores	Wang et al. (2000)
Bark	70% ethanol	3 min	Cut into pieces	0.1 ml bark paste added to 15 ml	Growth was observed. Individual hyphal tips of the various fungi were removed from the agar plates and placed on new PDA medium and incubated at 25 °C for at least 2 weeks. Checked for purity	Huang et al. (2001)
	Sterile water	Several times	0.5 gm bark pieces ground into paste with 2 ml sterilized water	PDA medium in a Petri plate and cultured at 25 °C		

Leaf (2 × 2 mm from leaf blade and 2 mm long from petiole)	70% ethanol	1 min		2% MEA plates. Incubated for 1 month at 20 °C	Fungi grown from the segments were recognized as endophytes and isolated for morphological identification	Hata and Sone (2008)
	15% hydrogen peroxide	15 min				
	70% ethanol	1 min				
Leaf (6 mm dia. disc of veins and interveins), petiole (6 mm disc from 3–4 cm long segments)	75% ethanol	1 min		Surface dried and placed on 90 mm Petri dish containing 2% MEA, supplemented with 1 mg/ml streptomycin sulphate and 0 ± 03 mg/ml rose bengal	Incubated at 25 °C for 2 months. Developed colonies transferred to new plates with MEA. Subcultures done with PDA, CMA, TWA	Guo et al. (2000)
	65% commercial Chlorox (3.25% aqueous sodium hypochlorite)	10 min				
	75% ethanol	30 sec				
Leaves, stems and fruits (0.5 cm^2)	Running tap water	Thorough wash		Nutrient agar	Incubated at 23–25 °C for 10 days. Bacteria emerging from tissue were purified and cultured on NA plates	Shweta et al. (2013)
	70% (v/v) ethanol	3 min				
	4% sodium hypochlorite solution	3 min				
	Sterile water	3 times				
Leaves, branch pieces	Running water	Thorough wash		1.5% oxoid malt extract agar (MEA) supplemented with 250 mg/L Terramycin to suppress bacterial growth	Incubated at 20 ± 2 °C for 5–14 days. Isolation was by transfer of mycelium, conidia or ascospores to 2% MEA plates	Fisher et al. (1993)
	75% ethanol	1 min				
	0.93–1.3 M sodium hypochlorite	3 min				
	75% ethanol	0.5 min				

To selectively isolate endophytes of interest, i.e. either bacteria or fungi, growth inhibitory compounds are added to the isolation medium which preferentially permits the growth of only the organism of interest while restricting the unwanted organisms. For example, nalidixic acid and nystatin are supplemented in the isolation media to selectively isolate actinomycetes (Gohain et al. 2015). Similarly, antibacterials such as chloramphenicol can be added to facilitate growth of only endophytic fungi, by avoiding endophytic bacteria (Melo et al. 2014), while streptomycin can be used to isolate fungi with slower growth rate (Miller et al. 2012a, b). Morphologically distinct colonies obtained from the explants incubated on an isolation medium are further isolated and purified to obtain pure culture. Further, the pure cultures are screened for their ability to produce various metabolites or bioactive compounds. Various surface sterilization protocol and the specially formulated medium used for the isolation of endophytes from literature are provided in Table 17.3.

17.6.2 Screening of Endophytes for Valuable Metabolite Production

This step involves screening of the endophytes based on their ability to produce diverse bioactive compounds. Many endophytes are known to produce growth inhibitory compounds (such as antibiotic, antibacterial, antifungal) which can be of commercial interest. Such endophytes can be screened by their ability to inhibit test strains grown on the same agar plate. Alternatively, the spent medium, i.e. the fermentation broth used to grow the endophyte, can be used to check for its growth inhibitory potential using a test organism. For example, endophytic isolates (*Streptomyces* sp., *Streptosporangium* sp. and *Nocardia* sp.) from *Azadirachta indica* showed inhibitory effect on *Pseudomonas fluorescens*, *E. coli*, *S. aureus*, *B. subtilis*, *C. albicans*, *Microsporum* sp., *Phytophthora* sp., *Trichophyton* sp., *Aspergillus* sp. and *Pythium* sp. Methanolic extracts of the isolates' spent media were infused on paper discs, and the assay was performed by using Bauer-Kirby method with slight modifications (Verma et al. 2009). Similarly, methanolic extract from the spent media of *Colletotrichum gloeosporioides* isolated from *Vitex negundo* showed inhibitory effect on *B. subtilis* MTCC 619, *P. aeruginosa* MTCC 2488, *S. aureus* MTCC 3160, *E. coli* MTCC 4296 and *C. albicans* MTCC 3018, when used individually. Interestingly, the same extract if used in synergistic combination with antibiotics (vancomycin and penicillin) showed better inhibitory effect on the multidrug-resistant *S. aureus* strain 6 (Arivudainambi et al. 2011).

The antiparasitic activity of the extracts can be tested by performing gGAPDH and APRT assays. For example, *Diaporthe phaseolorum* isolated from *Viguiera arenaria* showed inhibition of GAPDH enzyme and adenine phosphoribosyltransferase (APRT) enzyme. The fermentation broth of the endophyte was extracted with ethyl

acetate, and it was found to inhibit gGAPDH enzyme of *Trypanosoma cruzi* by 95% and APRT enzyme of *Leishmania tarentolae* by 60.7% (Guimarães et al. 2008).

Similarly, the ability of endophytes to produce industrially relevant enzymes is screened by plating them on agar plates with suitable substrates. For example, skimmed milk agar plates are used to evaluate protease activity, carboxymethylcellulose (CMC) agar plates are used to determine cellulase activity, and chitin agar (CA) plates are utilized to evaluate the chitinase activity of the endophytes by measuring their zone of inhibition (Zheng et al. 2011). These methods of screening are generally used when the endophytes are screened for untargeted compounds. Few examples of similar activity screenings reported earlier have been listed in Table 17.4.

17.6.3 Extraction of Metabolites from Endophytes

Isolation of endophytic fungi is relatively a simple process; however screening them for the presence of metabolite is often complicated, especially in case of discovery of novel compounds which is quite challenging process. It is often straightforward to identify a class of compound but difficult to narrow down to a precise one. A wide range of the solvents have been employed in literature to extract out the metabolite of interest from the endophytes. It should be considered that a metabolite can be extracellular or intracellular. In few cases, metabolites are seen to be observed both in the culture medium and the cell pellet. *Gibberella fujikuroi* MTCC 11382 isolated from *Amoora rohituka* bark produced 1.93 μg/gm of rohitukine from the mycelia and 0.72 μg/mL rohitukine from broth (Kumara et al. 2014). Extracellular metabolites are generally present in the medium and can be extracted by simple liquid-liquid extraction method. Rohitukine was extracted from the spent media twice by using equal volume of n-butanol in a separating funnel (Kumara et al. 2014). On the other hand, intracellular metabolite requires cell disruption techniques to bring the metabolites from the cells into the solvent. Various cell disruption techniques such as homogenizer and sonicator are conventionally used. In a recent report, camptothecin was extracted from *Fusarium solani* MTCC 9668 by sonicating the dried biomass suspended in water using an ultrasonicator (Venugopalan et al. 2016). Similarly in another report, homogenization using a mortar and pestle was employed for disruption of cell wall (Shweta et al. 2013). Along with the conventional methods, microwave-assisted extraction has also been employed for camptothecin and is found to give better product yield when compared with the conventional methods (Fulzele and Satdive 2005). However, for purification of the product from crude mixture, solvent extraction plays a major role, which is mainly selected based on the polarity of the compound of interest. Solvents should have optimum polarity to dissolve both polar and non-polar compounds. Therefore, usage of very alkaline or acidic and extremely polar solvent should be evaded (Milne et al. 2013).

Table 17.4 Representative list of bioactivity of endophytes against various test strains

Endophytes	Activity	Host plant	Type of extract	Test strain	References
Bacillus tequilensis, *Chryseobacterium indologenes*, *Pseudomonas entomophila* and *Bacillus aerophilus*	Antibacterial; antifungal	*Aloe vera*	Crude and ethyl acetate	*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus pyogenes* and *Candida albicans*	Akinsanya et al. (2015)
Streptomyces sp., *Streptosporangium* sp. and *Nocardia* sp.	Antibacterial; antifungal	*Azadirachta indica*	Methanol	*Pseudomonas fluorescens*, *S. aureus*, *E. coli*, *B. subtilis*, *C. albicans*, *Trichophyton* sp., *Microsporum* sp., *Aspergillus* sp., *Pythium* sp. and *Phytophthora* sp.	Verma et al. (2009)
Colletotrichum sp., *Fusarium* sp., *Guignardia* sp., *Phomopsis* sp., *Phoma* sp. and *Microdochium* sp.	Antibacterial	*Tradescantia spathacea*	Ethyl acetate	*P. aeruginosa*, *S. aureus* and *E. coli*	Alvin et al. (2016)
Diaporthe phaseolorum	Antiprotozoan	*Viguiera arenaria*	Ethyl acetate	*Trypanosoma cruzi*, *Leishmania tarentolae*	Guimarães et al. (2008)
Macrophomina phaseolina	Antifungal	*Ocimum sanctum*	Hexane	*Sclerotinia sclerotiorum*	Chowdhary and Kaushik (2015)
Botryosphaeria dothidea, *Fusarium proliferatum*, *Rhizopus* sp. and *Aschersonia* sp.	Antibacterial; antifungal	*Camptotheca acuminata*	Supernatants	*B. subtilis*, *E. coli*, *Fusarium solani* and *Verticillium dahliae*	Machavariani et al. (2014)
Nocardia caishijiensis	Antibacterial; antifungal	*Sonchus oleraceus*	Crude	*S. aureus*, *E. coli*, *K. pneumoniae*, *S. aureus* and *Candida tropicalis*	Tanvir et al. (2016)
Colletotrichum gloeosporioides	Antibacterial; antifungal	*Vitex negundo*	Methanol	*S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *C. albicans*	Arivudainambi et al. (2011)
Streptomyces sp.	Antibacterial; antifungal	*Polygonum cuspidatum*	Ethyl acetate	*E. coli*, *Salmonella* sp., *B. subtilis*, *Enterococcus faecium*, *S. aureus* and *C. albicans*	Wang et al. (2016)
Phoma sp.	Antifungal	*Eleusine coracana*	Methanol	*Fusarium graminearum*, *Fusarium lateritium*, *Fusarium sporotrichioides*, *Fusarium avenaceum*, *Trichoderma longibrachiatum*, *Aspergillus flavus* and *Alternaria alternata*	Mousa et al. (2015)
Pseudonocardia carboxydivorans	Antibacterial; antifungal	*Ageratum conyzoides*	Crude	*B. subtilis*, *C. tropicalis*, *S. aureus* and *E. coli*	Tanvir et al. (2016)

17.6.4 Identification and Confirmation of Metabolites from Endophytes

Preliminary investigation to test the presence of metabolites in the crude extracts from the culture broth of the endophytes involves techniques such as TLC (thin-layer chromatography), HPTLC (high-performance thin-layer chromatography) or HPLC (high-performance liquid chromatography) that gives information for the presence of a compound by matching with their standards.

Thin-layer chromatography (TLC) is a rapid technique using which multiple samples can be screened for the presence of metabolites. Crude extracts of multiple samples can be spotted on the silica plates along with the standards and drawn up using suitable solvents via capillary action. The plates are then visualized under UV with the presence of appropriate indicators, if needed. HPTLC was reported to quantify taxol content from 20 endophytic fungi samples by comparing with standard taxol using chloroform: methanol (9:1) as the solvent system. The samples exhibited many spots on the plate with one of them corresponding to standard taxol indicating its presence in the test sample (Gangadevi and Muthumary 2008). In another report, TLC and HPTLC were used to detect camptothecin content in the endophytic extract. The extracts along with the standard are spotted on the silica gel plates and developed using chloroform and ethyl acetate in the ratio 1:1 and analysed using TLC scanner and Win CATS 1.4.4.6337 software at a wavelength of 254 nm (Bhalkar et al. 2015, 2016).

HPLC is yet another most widely used technique for the identification and quantification of secondary metabolites. HPLCs are well known for their high reliability, accuracy, reproducibility and precision in data measurement. Small amount of the samples are separated on the column packed with 2–50 μm particles as stationary phase based on the difference in their physicochemical interactions and partition coefficients between the stationary and the mobile phase. Reduced flow rate and smaller pore-sized packing facilitate better separation with high precision and accuracy. The retention times of the analytes are compared with that of their standard retention time and quantified using the standard correlations built using X-Y plots of area under the curve versus known concentrations of the standard. HPLC-based quantification has been employed widely in literature for various secondary metabolites produced by endophytes like paclitaxel (Jianfeng et al. 1999; Pan et al. 2004; Renpeng et al. 2006; Sun et al. 2008; Deng et al. 2009; Liu et al. 2009; Zhang et al. 2009; Kumaran et al. 2010), camptothecin (Amna et al. 2006; Rehman et al. 2008; Kusari et al. 2009b; Gurudatt et al. 2010; Shweta et al. 2010; Pu et al. 2013), vinca alkaloids (Guo et al. 1998; Yang et al. 2004; Yin and Sun 2011), azadirachtin (Kusari et al. 2012b), podophyllotoxin (Eyberger et al. 2006; Puri et al. 2006; Cao et al. 2007; Kour et al. 2008), rohitukine (Kumara et al. 2014), etc.

However, the above said methods do not confirm for the presence of the compound when standards are not available. However, mass spectrometry is a tool for identification of known and unknown compounds and for confirmation of specific targeted compounds. Coupling of mass spectrometry with liquid and gas chromatography is a powerful technique for detection and identification of low-volume known and novel compounds in crude extracts.

The endophyte screened for the presence of bioactivity can be further subjected to gas chromatography-mass spectrometry (GC-MS) to identify the array of compounds present in the extract which can be responsible for the bioactivity. GC-MS approach helps us in identifying the compounds by performing a library search from the databases (Schauer et al. 2005). The mass of the compounds corresponding to each peak of the chromatogram is further fragmented into MS^2 and compared with the library to predict and identify the compound. Stoppacher et al. (2010) reported identification of 25 different microbial volatile organic compounds (2-heptanone, 1-octen-3-ol, 3-octanone, 2-pentyl furan, 3-octanol, α-phellandrene, α-terpinene, β-phellandrene, γ-terpinene, α-terpinolene, 2-nonanone, phenylethyl alcohol, 2-n-heptylfuran, p-menth-2-en-7-ol, 2-undecanone, α-bergamotene, β-farnesene, 6-pentyl-α-pyrone, γ-curcumene, α-curcumene, α-zingiberene, α-farnesene, β-bisabolene, β-sesquiphellandrene, nerolidol) from the extract of *Trichoderma* sp. by coupling solid-phase extraction with GC-MS. Similarly, extract of an endophyte *Colletotrichum gloeosporioides* isolated from *Lannea corammendalica*, when subjected to GC-MS analysis, displayed the presence of compounds such as 9-octadecenamide, hexadecanamide, diethyl pythalate, 2-methyl-3-methyl-3-hexene and 3-ethyl-2,4-dimethyi-pentane and exhibited antimicrobial activity. Another strain of *C. gloeosporioides* isolated from *Phlogacanthus thyrsiflorus* revealed the presence of phenol, 2,4-bis (1,1-dimethylethyl), 1-hexadecene, 1-hexadecanol, hexadecanoic acid, octadecanoic acid methyl ester and 1-nonadecene upon GC-MS analysis (Rabha et al. 2015). It is to be noted that gas chromatography can be employed only for compounds which could be volatile and thermostable.

Liquid chromatography-mass spectrometry (LC-MS) approach is majorly used for specific metabolite confirmation since they lack inbuilt library search as in the case of GC-MS but has higher resolution and sensitivity. Recent advanced versions of LC-MS instrument with high accuracy have the capability to display $[M + H]^+$ value up to 4 decimals with an error of less than 5 ppm. Additionally, fragmentation of specific *m/z* values results in MS/MS ion formation, which can be compared with literature for further confirmation. Few $[M + H]^+$ values and their MS/MS fragments of known metabolites are shown below in Table 17.5. There are also several online search tools or databases such as the METLIN database (Smith 2005), the Madison Metabolomics Consortium Database (MMDB) (Cui et al. 2008) and the Human Metabolome Database (HMDB) (Wishart et al. 2009). These databases help in identification by comparing the spectral data with those available from the databases for metabolite search. However these databases are yet to be updated with many compounds which are still unreported (Vasundhara et al. 2016). Though mass spectrometry confirms our metabolite at molecular weight level, isomers which have varying structures cannot be clearly differentiated with this technique.

Nuclear magnetic resonance (NMR) is an approach which helps in structure prediction of known or novel compounds and also confirmation of known compounds by analysing the proton (^1H) or carbon (^{13}C) magnetic resonance. For example, presence of vincristine and vinblastine in the endophytic extract was confirmed by analysing the ^1H NMR spectra and chemical shift of the endophytic vincristine and vinblastine in comparison with the standards (Kumar et al. 2013). Similarly,

Table 17.5 m/z values of selective metabolites and their fragmentation pattern.

Compound	$[M + H]^+$	MS^2 fragments	References
Azadirachtin	663	645, 627, 609, 545, 527	Kusari et al. (2012b)
Camptothecin	349.1	305, 447.3, 284.2, 149.0	Ramesha et al. (2008) and Shweta et al. (2010)
9-Methoxy camptothecin	379.1	335.2, 516.4, 474.3, 305, 379.2	
10-Hydroxy camptothecin	365.1	303, 305	
Diacetoxy-camptothecin	431.1	349.1, 303, 149	Ramesha et al. (2008)
Diacetoxy-9-methoxy camptothecin	461.2	379.1, 333.1, 415.2	
Acetoxy-camptothecin-glycoside	511.1	469.2, 365.1, 289.0, 307.1, 349.1, 149, 189	
9-Methoxy-mappacine-20-β-glucopyranoside	499.2	337.1	
Mappicine-20-β-glucopyranoside	469.2	289, 307, 365.1, 207, 349, 319	
Rohitukine	306.1	288, 245	Kumara et al. (2014)
Rohitukine N-oxide	322.12	304, 276	
Piperine	286.1	135, 143, 171, 201	Chithra et al. (2014)
Paclitaxel	854.3	286, 367, 395, 464, 509, 545, 551, 568, 587	Das et al. (2017)
Vinblastine	811	355, 522, 542, 733, 751, 793	Kumar et al. (2013)
Vincristine	825	766, 807	

withanolide from the endophyte *Talaromyces pinophilus* isolated from *Withania somnifera* was structurally confirmed using NMR (Sathiyabama and Parthasarathy 2017). However, convectional NMR requires metabolite to be in the pure form for structure prediction. Recent development such as coupling an LC with NMR has made that task even simpler, where separation can be made by the LC and the fractions can be analysed simultaneously in NMR. Though LC-NMR helps in analysis of each peak of the chromatogram using a stop flow valve, it is difficult to analyse crude extract, with complex and closely eluting compounds (Wolfender et al. 2001). An overall representation of various techniques used for identification and quantification of targeted and untargeted metabolites is given below (Fig. 17.2).

17.7 Bioprocess Optimization Strategies for Enhanced Metabolite Production by Endophytes

17.7.1 Culture Condition Optimization

Fermentation parameters such as temperature, pH, medium composition, agitation, inoculum concentration and photoperiod are known to significantly affect the yield of secondary metabolites in fermentation processes (Thiry and Cingolani 2002). A

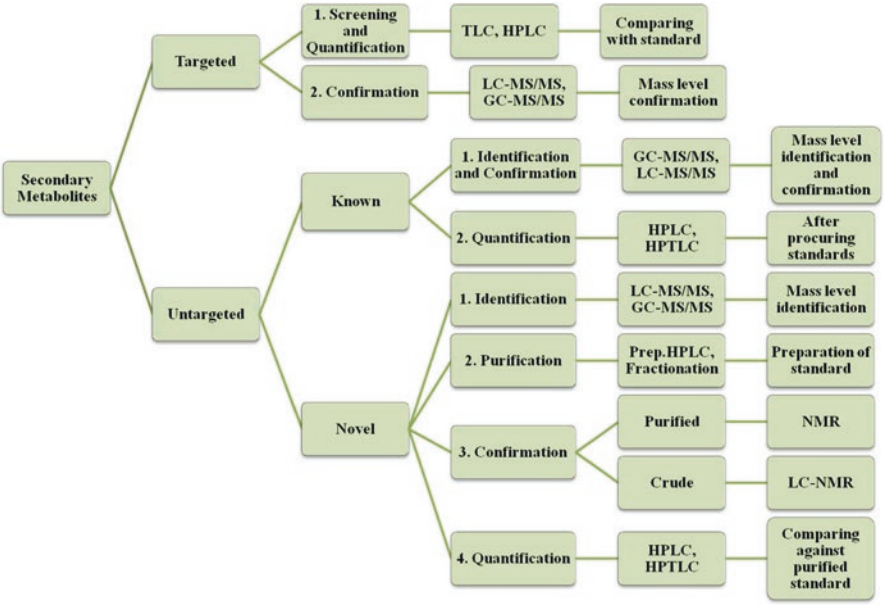

Fig. 17.2 Schematic representation of the steps which can be involved in the identification and quantification of known and novel metabolites produced in endophytic fermentation

straightforward approach to culture condition optimization is single-factor optimization, where each factor is separately optimized while keeping all other factors constant. However, statistical optimization gives us the advantage of understanding interactions between different factors with minimum number of experiments.

In the case of endophytic fungi, statistical as well as single-factor optimization has been explored. An eightfold increase was observed in the yield of zofimarin (antifungal compound) from the endophyte *Xylaria* sp. Acra L38 after statistical optimization of carbon and nitrogen sources (Chaichanan et al. 2014). Similarly, a single-factor medium optimization study resulted in 10.3-fold enhancement in the production of mycoepoxydiene from the endophyte *Phomopsis* sp. Hant25 (Thammajaruk et al. 2011). Optimization of initial pH along with carbon and nitrogen sources resulted in 1.27-fold enhancement in beauvericin production from *Fusarium redolens* Dzf12 (Xu et al. 2010). In another study optimization of temperature and medium composition gave 77% enhanced sipeimine yield from *Fritillaria ussuriensis* Fu7 (Yin and Chen 2011).

17.7.2 Exogenous Additions

17.7.2.1 Elicitors

Elicitors are molecules that are involved in signalling under different stress conditions such as pathogenesis and hypersensitivity and are known to induce and regulate many genes. Elicitors may be classified as biotic and abiotic, and abiotic

elicitors may be further classified as physical and chemical. Biotic elicitors include materials of biological origin such as chitin, polysaccharides, glycoproteins, etc.

Elicitors such as salicylic acid and methyl jasmonate are signalling molecules in plant systems and have been widely used for yield enhancement even in endophytic systems. Among other chemicals used as elicitors, metal ions are involved in metabolism indirectly as enzyme cofactors or directly by means of redox reactions with other metabolites. Hence, metal ions can be added as a means to enhance the yield of target metabolites. Liu et al. (2010) optimized the production of 10-hydroxycamptothecin in *Xylaria* sp. isolated from *Camptotheca acuminata* by adding various elicitors, which included metal ions such as Ce^{3+}, Cr^{3+}, La^{3+}, Cu^{2+}, Fe^{2+}, Se^{5+}, Mn^{2+}, Ca^{2+} and Li^{+}. Among them Mn^{2+} and Li^{+} produced a yield of 5 mg/l compared to the control (2 mg/l).

Somjaipeng et al. (2016) studied the effect of seven different chemical elicitors (salicylic acid, jasmonic acid, phenylalanine, serine, silver nitrate, sodium acetate and ammonium acetate) on taxol yield from the endophytes *Paraconiothyrium variabile* and *Epicoccum nigrum*. They also studied the synergistic effects of the elicitor and the pH of the growth medium using response surface models, which is one of the statistical optimization methods commonly used in bioprocess optimization. Serine was found to be the best elicitor for *E. nigrum*, resulting in an increase of taxol yield up to 29.6-fold.

In another study by Qiao et al. (2017), taxol yield from the endophytic fungus *Aspergillus aculeatinus* Tax-6 isolated from the tree *Taxus chinensis* was improved from 335 μg/L to 1338 μg/L after addition of sodium acetate, salicylic acid and copper sulphate. Copper ions are said to increase the activity of oxidases involved in taxol biosynthesis, and salicylic acid is a well-known signalling molecule that acts as an elicitor. The amounts of the elicitors added were further optimized using response surface methodology.

17.7.2.2 Precursors

Another strategy to improve the product yield is by exogenously adding its biosynthetic precursors in the culture medium. Adding intermediates of the desired metabolite synthesis pathway can increase the reaction flux towards the desired metabolite leading to its enhanced production. Such intermediates may be readily available and hence this technique is useful. The amount of precursor added must be optimized such that there is an increase in yield without causing toxicity to cells (Gaosheng and Jingming 2012). Amna et al. (2012) reported the stimulation of camptothecin production from the endophytic fungus *Entrophospora infrequens* RJMEF001 using various precursors such as tryptophan, tryptamine and leucine.

Apart from elicitors and precursors, enzyme inhibitors such as 5-azacytidine, which blocks DNA methyltransferase, have been added exogenously to sustain the production of secondary metabolites. As DNA methylation was hypothesized to attenuate camptothecin production in the endophyte by silencing the genes involved in camptothecin biosynthesis, this enzyme inhibitor was used to enhance camptothecin production in the attenuated cultures of the endophyte *Botryosphaeria rhodina* isolated from *Camptotheca acuminata* (Vasanthakumari et al. 2015). Also, multiple

strategies mentioned above may be combined to produce a synergistic effect as observed by Pu et al. (2013) in the case of camptothecin production from the endophyte *Trichoderma atroviride* isolated from *Camptotheca acuminata.* A combination of different optimization strategies involving culture conditions (media composition, pH, temperature, agitation, incubation time) and elicitation led to a 50-fold enhancement in the yield of camptothecin.

17.7.3 Co-cultivation

Several strategies in the case of optimization of secondary metabolite production seek to simulate the natural environment of the endophyte. The regulation of biosynthetic genes is tightly linked to environmental parameters, and hence secondary metabolites are produced only when required by the cells. Co-cultivation of endophytes with other endophytes or cells/tissues from the host plants is one such strategy that seeks to simulate the natural environmental conditions. In several cases, a significant yield enhancement has been reported with the use of co-cultivation strategy.

The parameters such as inoculum ratio, environmental parameters, medium components and reactor design can be optimized during co-cultivation to maximize the production of the desired metabolite (Venugopalan and Srivastava 2015).

17.7.3.1 Microbial Co-culture Systems

In the natural environment of the endophyte, it also interacts with other endophytes and invading microorganisms which may also affect the metabolite production by the endophyte. It is hence worthwhile to experiment with co-cultures of different endophytes to enhance the production of secondary metabolites.

Soliman and Raizada (2013) worked with the taxol-producing endophyte *Paraconiothyrium* SSM001 and reported that co-culturing the endophyte with another endophyte *Alternaria* sp. resulted in a threefold increase in taxol yield. Further, adding another endophyte *Phomopsis* sp. to this co-culture system resulted in a net eightfold increase in taxol production. They hypothesize that *Paraconiothyrium* SSM001 produces more taxol in response to other fungi that invade the plant, so as to benefit the plant and survive in symbiosis with the plant.

Ola et al. (2013) reported a 78-fold increase in the yield of enniatin A1 from the endophyte *Fusarium tricinctum* when co-cultured with *Bacillus subtilis* in comparison to axenic culture. They also observed that some metabolites that were not detected in the axenic culture were found to be above the detectable limits in the co-culture system. Though there have been no reactor level studies reporting co-culture of an endophyte with another endophyte, it is possible to culture several strains in a bioreactor. For example, Hernández et al. (2018) cultivated up to four strains of fungi together in a batch process, for cellulase production. Similar approaches could be used to co-cultivate endophytic fungi for maximizing the yields of desired metabolites.

17.7.3.2 Plant-Endophyte Co-culture Systems

In nature, the endophytic fungi adapt to grow inside their host plants, and hence the profile of the metabolites can significantly change under axenic culture conditions possibly due to loss in in planta selection pressure and stimulus, thereby also affecting its biosynthetic potential. Therefore, one of the ways to simulate the natural environment under in vitro conditions can be by co-cultivation of plant cells/tissues with endophytes which may mutually benefit the two organism's (i.e. plant and microbe) biosynthetic capabilities. Ding et al. (2017) isolated three endophytic fungal strains, *Aspergillus* sp., *Fusarium* sp. and *Ramularia* sp., from the plant *Rumex gmelini* Turcz (RGT). All three strains were capable of producing bioactive metabolites that were produced by their host plant. They reported an increase in the production of the bioactive secondary metabolites, chrysophaein, resveratrol, chrysophanol, emodin and physcion in the seedlings of the plant when co-cultured with the endophytic fungi. In another co-culture study by Baldi et al. (2008), it was found that co-culturing podophyllotoxin-producing plant cells from *Linum album* showed an increased production of podophyllotoxin and 6-methoxypodophyllotoxin when co-cultivated with arbuscular-mycorrhiza like fungi. Co-culturing of *Linum album* plant cells with *Piriformospora indica* resulted in a yield enhancement of 3.6 times for podophyllotoxin and 7.4 times for 6-methoxypodophyllotoxin. Similarly, the same plant cells when co-cultivated with *Sebacina vermifera* resulted in an yield enhancement of 3.9 times for podophyllotoxin and 7.6 times for 6-methoxypodophyllotoxin These findings highlight that co-cultivation of endophytic fungi with plant cells when either or both of them are capable of producing the target metabolite can be a promising yield enhancement strategy.

Bioreactors for co-culturing plant cells and fungal cells have also been designed and reported in literature. Such bioreactors usually consist of two divisions, one each for plant and fungal cells, separated by a semipermeable membrane (Fig. 17.3). The semipermeable membrane serves for the exchange of metabolites between the plant cells and fungal cells, without having to place them in direct contact with each other. Li et al. (2009) co-cultured *Taxus chinensis* plant cells and the endophytic fungus *Fusarium mairei* isolated from the same plant in a specially designed bioreactor. The bioreactor consisted of two tanks, one each for the plant cell suspension and the fungus, separated by a membrane to allow only exchanges between metabolites (Fig. 17.3). A 38-fold increase in the pacilitaxel yield could be achieved in comparison to monocultures possibly due to exchange of metabolites (including biosynthetic intermediates) during the co-cultivation period.

17.7.4 Genetic Modifications

Genetic transformations can play a key role in commercial exploitation of endophytes, by enabling research on the genetics of endophytes as well as insertion of biosynthetic genes and regulatory elements of interest for yield enhancement of the target metabolite. One of the earliest methods used for transformation of fungal endophytes is protoplast transformation. PEG-mediated transformation and *Agrobacterium tumefaciens*-mediated transformations were also developed later.

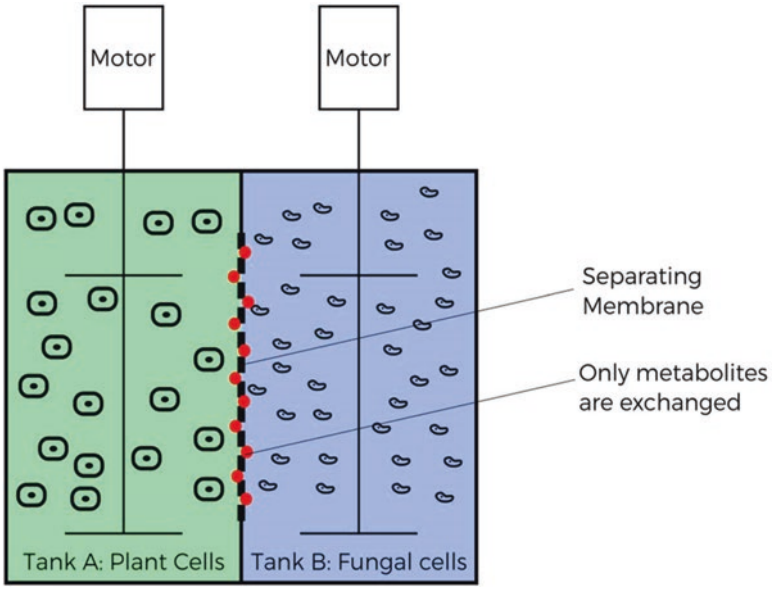

Fig. 17.3 Plant-microbe co-cultivation bioreactor set-up (adapted from Narayani and Srivastava 2017)

17.7.4.1 Protoplast Transformation

Protoplasts are cells in which the cell wall has been removed. Once the cell wall is removed, it is easier for the cells to take up exogenously added DNA. Protoplast transformation on endophytic fungi was first demonstrated by Long et al. (1998). A filamentous fungus, *Pestalotiopsis microspora*, isolated from the inner bark of the taxol-yielding Himalayan yew tree was used in the experiments. A gene-encoding hygromycin resistance was expressed using regulatory sequences from *Aspergillus*.

17.7.4.2 PEG-Mediated Protoplast Transformation

While using protoplast transformation, adding PEG (polyethylene glycol) increases the rate of uptake of DNA into cells and is hence used in transformation techniques. Wei et al. (2010) established a PEG-mediated transformation protocol for the endophytic fungal strain *Ozonium* sp. EFY-21. The strain is known to produce taxol. A gene conferring resistance to hygromycin was expressed the trpC promoter from *Aspergillus nidulans* to verify successful transformation. This protocol was used by the same group (Wei et al. 2012) to overexpress the taxadiene synthase gene in the same strain, *Ozonium* sp. EFY-21,which resulted in an increase of up to 3.77-fold in the taxol yield.

17.7.4.3 *Agrobacterium tumefaciens*-Mediated Transformation

Agrobacterium tumefaciens is commonly used for transformation of plant cells. Interestingly, Bundock et al. (1995) reported that *A. tumefaciens* is able to transfer its T-DNA to a fungal species, *Saccharomyces cerevisiae*. Several other reports on

using *A. tumefaciens* for transforming different types of fungi subsequently came out (Aimi et al. 2005; Michielse et al. 2005; Betts et al. 2007).

Liu et al. (2013) successfully used this method of transformation on the above-stated taxol-producing endophytic strain of *Ozonium* sp. EFY-21. The transformation efficiency was higher compared to the PEG-mediated transformation method. An *Agrobacterium*-mediated transformation protocol was used by Soliman et al. (2017) to integrate geranylgeranyl diphosphate synthase gene into the genome of the taxol-producing fungus *Paraconiothyrium* SSM001. Geranylgeranyl diphosphate is a precursor in taxol synthesis, and a threefold increase in taxol yield was observed when this precursor was overproduced by the modified fungal cells.

17.7.4.4 Nuclease-Based Methods: REMI and CRISPR

REMI (restriction enzyme-mediated integration) is a method of integrating DNA fragments into the host genome using restriction enzymes introduced into the cells. It was first demonstrated by Schiestl and Petes (1991). The taxol-producing endophytic strain *Ozonium* sp. BT2 was transformed using this method by Wang et al. (2007b) which was proven to have increased transformation efficiency when compared with conventional PEG-mediated protoplast transformation (Bölker et al. 1995). However, there are not many reports demonstrating the use of REMI on endophytes.

The CRISPR/Cas9 system from the bacterial adaptive defence system (Barrangou et al. 2007) has been adapted into a tool for a genome editing (Doudna and Charpentier 2014). There is a considerable potential for the use of CRISPR/Cas9-based genome editing in endophytes as the system offers simple customizability with regard to the target sequences and precision in editing. Though CRISPR/Cas9 has not been directly demonstrated on an endophyte after isolation from a plant system, a report by Chen et al. (2017) demonstrates the use of CRISPR/Cas9-based genome editing in the fungus *Beauveria bassiana*, which is capable of growing as a plant endophyte (Parsa et al. 2013). Apart from introducing biosynthetic genes into endophytes, CRISPR/Cas9 system may also be used to edit regulatory sequences and control the expression of biosynthetic genes that are not expressed under axenic conditions. Hence, we can expect CRISPR/Cas9-based genome editing to be used for genetic modification of endophytes in the future.

17.8 Conclusion and Future Directions

Endophytes continue to be a promising alternative source for production of plant-based secondary metabolites. Literature suggests that one of the major reasons for product yield attenuation in endophytes under axenic conditions could be the absence of genetic and epigenetic stimulus provided by the natural environment and lack of biosynthetic intermediates. Implementation of bioprocess optimization strategies has resulted in yield enhancement of secondary metabolites during endophytic fermentations. The product yield retrieval and enhancement even in the attenuated strains of endophytes via bioprocess optimization strategies demonstrate

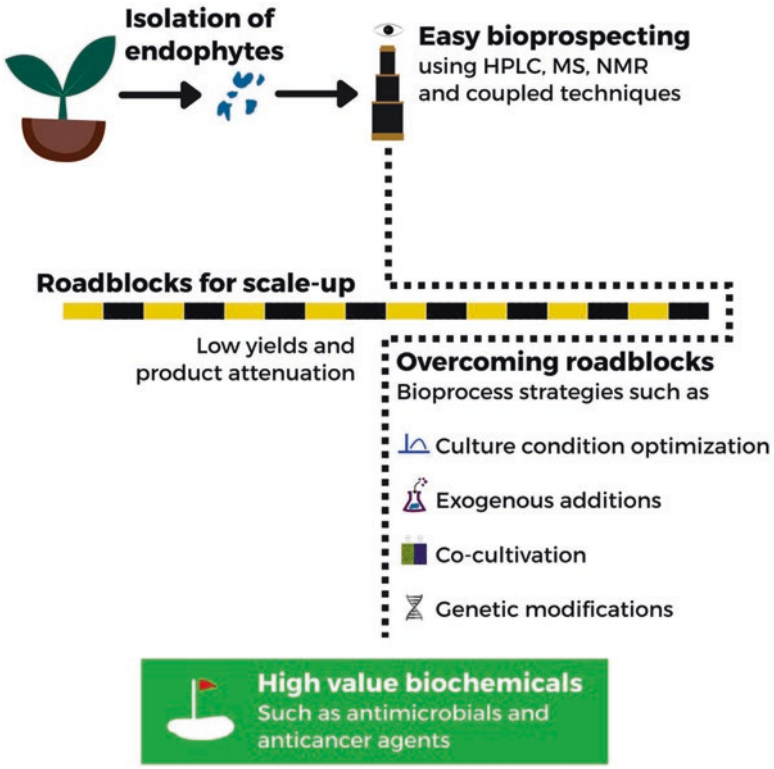

Fig. 17.4 Schematic summary of endophyte-based bioprocess development for in vitro production of high-value biochemicals

that endophytes are capable of metabolite production even under in vitro conditions if provided an optimum environment. Hence, mimicking the natural environment under in vitro condition and activation of silent genes through genetic modification in combination with the most optimum fermentation conditions (Fig. 17.4) can help us overcome the current limitation of low product yield and attenuation in endophyte fermentations.

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