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Microbial Paclitaxel: Strategies for Enhancing Paclitaxel Production at the Classical & Transcriptional Level with Genetic tools

Correspondence author's: Sandarbh Rishi[1], Dr. Syed Shahab Ahmed[2],

1. Research Scholar, Sri Satya Sai University of Technology & Medical Sciences, Sehore
2. Associate Prof., Sri Satya Sai University of Technology & Medical Sciences, Sehore

Abstract

Paclitaxel is a widely used chemotherapy drug typically Extracted from the Yew tree. Paclitaxel is a poly-oxygenated cyclic diterpenoids with a characteristic Taxane ring system. It is the most effective and widely used chemotherapeutic drug for the treatment of cancers and virus-related sarcoma. Paclitaxel has high activity as an anticancer agent and is widely used in hospitals and clinics. Paclitaxel is a naturally occurring chemical component that was first identified from the bark of yew trees but presents at low levels1. In recent years, lots of yew trees have been destroyed or seriously damaged by harvesting to extract paclitaxel. Although some fungi endophytic to yew have been found to produce paclitaxel, but the levels of production are too low to be useful for commercialization until now. To find endophytic fungi that can produce paclitaxel, we isolated and collected lot of endophytic fungal strains from Bhattu Buhla, Baijnath Tehsil in Kangra District Himachal Pradesh (India). Taxus Baccata distributing in Among these endophytic fungal strains, one strain that can produce paclitaxel was isolated and its production was authenticated with LC-MS. This discovery is very significant for paclitaxel manufacture and environmental protection of yew trees. Each year, approximately 8.1 million new cancer cases are diagnosed worldwide, including India. Around five hundred patients are treated by 1 kg of Paclitaxel, which requires 10 tons of bark equivalent to 300 trees. Too frequent extraction involves destroying the Yew tree source. Demand for Paclitaxel has outstripped supply, and scientists have turned to developing Paclitaxel in plant cells followed by industrial fermentation. Through Biotechnology and Genetic Engineering, we can be generating Paclitaxel in bacteria/Fungal cell cultures instead of plant cell cultures.

Key Words:- Taxus Baccata, Taxus Brevifolia, Aspergillus Fumigatus, Paclitaxel, HPLC, Cytochrome P-450, Plant Tissue Culture, 10-deacetylbaccatin III (10-DAB III), Endophytes.

INTRODUCTION

Paclitaxel is the most effective and widely used chemotherapeutic drug for the treatment of cancers and virus-related sarcoma. First time, Paclitaxel was isolated from the Taxus brevifolia (bark, roots, and branches). So far, the source of Paclitaxel is either semi-synthetic precursors like baccatin III and 10-deacetyl baccatin III or natural yew tree. Although the concentration of paclitaxel is very low (0.01 %–0.05 %) from the natural sources, still, the bark of yew (Taxus) is the principal source.

The high cost of the drug is attributed to the inadequate supply of natural paclitaxel and increasing application in chemotherapy. Recently, microbial fermentation technology emerged as an alternative approach for cheaper and higher yield of paclitaxel. In the past decades, numerous paclitaxel producing entophytic fungi have been isolated. However, none of them achieved an industrial production platform because of the low amount of paclitaxel production. However, due to the rapidly growing market, low availability, and the fact that Taxus spp. are rare, endangered and grow very slowly, an alternative source is needed to produce

paclitaxel at large commercial scale. Considering these factors, the current study was carried out on isolation, identification, and extracellular production of paclitaxel from Aspergillus Fumigatus isolated from Taxus sp. Therefore, researchers are looking for newer approaches using recombinant technology to improve the yield from isolated paclitaxel producing fungi, in addition to search novel high paclitaxel producing stable microbial isolates from nature. Prior research has already shown paclitaxel to be an effective chemical in combatting cancer. As per the chemotherapeutic activity this compound "binds to micro-tubules, which are important in cell division, and prevents the cancer cells from dividing properly. However, just because a product is useful does not mean supply has kept pace with rising demand. Paclitaxel is primarily obtained by extracting it from Yew trees, which naturally synthesize the product. Given the solvents and treatment necessary to do so, however, this approach also destroys the very same Yew trees in the process. As such, extraction is unlikely to achieve demand-supply equilibrium in the market. Researchers have since pivoted to modifying plant cell cultures to produce paclitaxel and other significant precursors found along the metabolic pathway. These plant cell cultures are in turn used in industrial processes designed to produce paclitaxel on a substantial scale. Even this, however, is not the most efficient solution to the current shortage the plant cell's complex infrastructure and subsequent energy needs have prevented the cell's resources from being fully directed towards paclitaxel production. Low product yield is typically the result.

The 2016 Duke University International Genetically Engineered Machine team has produced paclitaxel more efficiently, by using bacteria/fungal cell cultures rather than plant cells. The process of optimizing bacteria/fungi to produce a product for later industrial fermentation has already been demonstrated, but its application to paclitaxel has not. The IGEM team has worked on characterizing five enzymes involved in the natural process of paclitaxel production, and then merging them into one strain by genetically engineering the DNA of the bacteria/Fungi culture. At the end of this process, the bacteria/Fungi culture produces paclitaxel, with less energy expenditure than was required in plant cells and subsequently higher yield. But a more efficient process is meaningless if the means to boost market supply are not available, which requires cooperation with a biopharmaceutical company.

In 1993, Stierle, et al. reported a fungus from the bark of a Pacific yew tree (Taxus brevifolia) that was able to produce Paclitaxel, even when the fungus was grown as a pure culture. This finding stirred up interest of scientists in searching for Paclitaxel-producing fungi all over the world that promises a different approach to produce the compound. Fungal fermentation has been widely used in agriculture, pharmaceutical, and food industries for a long history. It is certainly a good practice for Paclitaxel production at the industrial scale. Advantages of growing fungi over plant cell culture include the medium is simple and inexpensive, the period of fermentation is short, and fungi are convenient for genetic manipulation which is a benefit for breeding to improve the yield of Paclitaxel. Still more, since large-scale fermentation will provide sufficient biomass for Paclitaxel preparation, consumption of yew trees will eventually be eliminated.

Paclitaxel is still a high-priced drug, consequently out of reach for many people in the world. New approaches and further improvement of established production technology are needed to reduce the cost of Paclitaxel production. Particularly, isolation and identification of paclitaxel -producing endo-phytic fungus is a very prospective and feasible approach for the production of a large amount of paclitaxel. Paclitaxel has been detected in more than 30 unrelated fungal endophytes including the genera Pestalotia, Pestalotiopsis, Fusarium, Alternaria, Aspergillus Fumigatus, Pithomyces, Monochaetia, Penicillum, Truncatella, Tubercularia, Sporormia, Trichothecium and Seimatoantlerium (Strobel et al. 1996; Wang.et al. 2000; Shrestha et al. 2001). Fungi have a considerable potential to be used in economical and environmentally friendly fermentation processes for Paclitaxel production. They grow fast in simple culture media, can be manipulated easily and cultured on large scale. Consequently, fungi could provide a reliable source of Paclitaxel through fermentation. However, little is known about most of these fungi, and studies on the factors that control Paclitaxel production are limited.

Development of novel variant treatments utilizing Paclitaxel has boosted the consumption of Paclitaxel, and as a consequence the demand of Paclitaxel supply in the market keeps growing. Major obstacles for best therapeutic results with Paclitaxel are its poor solubility and consequently difficulties in delivery. Researchers have made attempts to improve the Paclitaxel efficiency by combination with other drugs or modified to develop Paclitaxel analogues Docetaxel (Taxotere), a modified derivative of Paclitaxel, is easier to formulate and administer due to its greater aqueous solubility.

Besides producing anticancer drugs in homologous and heterologous plant tissue cultures, production of plant-derived secondary metabolites in microbial hosts is a promising avenue despite several challenges. Taxane diterpenoids could become the first case among all the plant-derived anticancer drugs that are produced in microbial hosts because of their relatively well-characterized pathways. The biggest issue is how to efficiently express plant cytochrome
P450 enzymes in microbes, such as E. coli and S. cerevisiae. Ajikumar et al. have applied the transmembrane engineering strategy and generated chimera enzymes composed of P450 and
CYP450-reductase (CPR) to convert taxadiene to taxadien-5α-ol. Cytochrome b5 reportedly enhances the activity of certain cytochrome P450s. Therefore, coexpression of P450s together with CPR and cytochrome b5 may increase the enzyme activity.

However, isolation from natural sources is hindered not only by the slow growth of the Pacific Yew tree but by the low concentration of the drug in its bark, only 100 mg of Paclitaxel per kg of Bark (Bocca, 1998). Interest in Paclitaxel as a drug was low until the discovery of its mechanism in 1979, with clinical trials beginning in 1983 (Donehower, 1996). Paclitaxel was first approved for second line ovarian cancer treatment starting in 1992 and later for first line treatment starting in 1998 (Pai-Scherf). It has also been approved for breast, adenocarcinoma of the pancreas and non-small cell lung cancers (Food and Drug Administration, 2013).

Paclitaxel is small molecule metabolite that has been shown to work on many forms of cancer (Kingston, 2007). Paclitaxel promotes the formation of microtubule assembly in cells which disrupts tubulin and creates cytotoxicity (Schiff, Fant, & Horwitz, 1979). Further, even synthetic variations on the Paclitaxel structure have been tested and shown to have chemical activity, such as docetaxel (Kingston, 2007). The efficacy of the drug in treating various cancers has created a boom in demand for Paclitaxel and a supply shortage to keep up. In order to understand the current state of Paclitaxel production and the need for it today, this section will outline the history of Paclitaxel discovery, usage and synthesis to understand the market as it is now. Phyton has invested more than 200 man-years of effort to address the commercial issues of plant cell culture technology. Under a long-term collaborative relationship with Bristol-Myers Squibb Company (BMS), Phyton has developed and is commercializing a plant cell culture process to produce paclitaxel (the active ingredient of Paclitaxel; Paclitaxel is a registered trademark of BMS). Through its wholly owned subsidiary, Phyton Gesellschaft für Biotechnik mbH (Phyton GmbH), the Company operates the world's largest dedicated plant cell culture production facility in Germany. It is being used for paclitaxel production under current Good Manufacturing Practice (cGMP) conditions.

To date, much of the work on plant cell cultures that has translated into commercial success involves optimization strategies like those developed for other cell culture and fermentation processes. This type of process engineering approach includes manipulation of culture operating parameters such as media composition, cell line selection, and gas phase composition (reviewed in Kieran et al. 1997; Roberts and Shuler 1997). These strategies are a necessary starting point in many cases, especially when initiating a new cell line. The following section presents a brief overview and recent applications of some approaches that have traditionally been of interest to plant cell culture process development. While many of these ideas may be considered outdated in favor of newer metabolic engineering perspectives, their successful application is essential for the eventual success of plant cell culture technology, and therefore remain active areas of research.

Perhaps the most notable strategy for improving metabolite yields is elicitation. An elicitor can be defined as any compound that induces an up regulation of genes. Some elicitors target secondary metabolic genes, which are often associated with defence responses to perceived environmental changes. Elicitors include natural hormones, nutrients, and many fungi-derived compounds. Occurring hormones involved in the regulation of defence genes as part of a signal transduction system (Gundlach et al. 1992). Applied exogenously, they have been shown to induce secondary metabolic activity and promote accumulation of desired metabolites in numerous plant systems, including Taxus spp. (Mirjalili and Linden 1996; Yukimune et al. 1996) and C. roseus (Aerts et al. 1994; Lee Parsons and Royce 2006). For instance, MJ elicitation compared to salicylic acid elicitation in Taxus spp. cultures resulted in different relative increases of metabolic intermediates (Ketchum et al. 1999; Wang et al. 2004), suggesting that each elicitor preferentially directs flux towards, and possibly away from, and different intermediate taxanes. While many of the specific targets of elicitors have

yet to be conclusively identified, elicitation can be an extremely useful tool in conjunction with gene expression profiling for identifying rate influencing steps in secondary biosynthetic pathways.

Plant cells are much larger than mammalian cells or microbes which make them extremely susceptible to shear forces in the surrounding fluid. Different types of plant cells exhibit different responses related to shear forces, and detailed studies have been performed on individual species evaluating a variety of effects relative to shear forces, including reduction in viability, release of intracellular components, changes in metabolism, and changes in morphology (Zhong 2002).

Current Methods for Paclitaxel Production

Extraction of Paclitaxel from Yew Tree Materials: In the early years after marketed, Paclitaxel was largely extracted from wild yew trees, its bark and other parts. Leading pharmaceutical companies soon started large-scale farming of yew trees. By now, extract of this chemical from raw tree materials is still playing a part in Paclitaxel industry. Unfortunately, Paclitaxel makes up only a small proportion of the total taxoids in Taxus trees. Its natural concentration is approximately 0.01% of a dry weight basis in Pacific yew. The Taxane contents varied among and within Taxus spp. Even in Taxus media Hicksii, a hybrid variety of yew with a highest content of Paclitaxel known so far, Paclitaxel content is between 109 and 112 mg/kg of the dried powder. The problem with this extraction strategy is that Paclitaxel content is significantly influenced by the geographical location, altitude, age, and sex of the trees. The season to harvest and the way to handle the tree materials also have effects on the yield of Paclitaxel. Due to slow growth of yew trees and the low-yield extraction techniques, global production of Paclitaxel with this strategy is limited and cannot meet the growing market.

Paclitaxel from Plant Tissue Culture and Cell Suspensions:

Plant cell, tissue cultures, or cell suspension culture of Taxus spp. is considered as a comparably fast way to obtain sufficient amount of tree biomass. Taxus cell cultures have been extensively investigated to increase the content of Paclitaxel via different methods, including screening for the high-Paclitaxel-producing genotypes and hybrids, nutrients, chemical elicitors (methyl jasmonate, silver thiosulfate), plant growth regulators, the heat shock, mechanical stimulating, two-phase culture, and others. The production of Paclitaxel was remarkably improved by these processes. Plant cell fermentation (PCF) technology developed by the German and Canadian biotechnology company Phyton Biotech, Inc., is used for Paclitaxel production. However, low and unstable Paclitaxel yield, high production cost, and by-product contamination are the main bottleneck for large-scale commercial application of this technology. The overall cost of Paclitaxel production via cell culture or cell suspension remains still high.

Total Chemical Synthesis of Paclitaxel:

Several different synthesis routes of paclitaxel have been published. The total synthesis has been first achieved independently by two teams in 1994, the Holton's and the Nicolaou's. The third synthetic route was accomplished by Danishefsky's group in 1996. Total synthesis of Paclitaxel is a complex task for chemists given the fact that the molecule consists of four complicated rings (A, B, C rings and the oxetane ring) and has 11 chiral centres. The synthesis processed more than 20 steps, and only 0.07% and 2.7% production rates for the Holton's and Nicolaou's routes were obtained, respectively. Mukaiyama et al. have proposed an improved method for the asymmetric total synthesis of Paclitaxel by a different way. An automated synthesizer with a 36-step synthesis sequences for intermediate of Paclitaxel was developed by Doi et al. (2006). Due to the complexity of Paclitaxel structure, the expensive chemical reagents, and the strict requirement for reaction, the process of the total synthesis of Paclitaxel is multiple-step, costly, and commercially unfeasible for industrial application for Paclitaxel manufacture.

Semi synthesis of Paclitaxel:

Starting with 10-deacetylbaccatin III (10-DAB III), a precursor of Paclitaxel biosynthesis in yew with higher content, and several coupling strategies of a phenylisoserine moiety with protected 10-DAB III have been reported by several groups. 10-DAB III can be isolated from the needles of T. media and T. baccata with relatively higher yield. The development of Paclitaxel synthesized from baccatin III and C-13 Paclitaxel side chain represents a breakthrough of Paclitaxel industrial production and has become a major source of Paclitaxel and Taxotere in industry. This semi synthesis has significantly improved the production and global

supply of the drug and makes the drug more affordable to patients. However, an unsolved issue of this widely used method is its dependence on the supply of yew tree materials.

It seems evident that fungal fermentation has advantages over the current techniques that are currently used in industrial production of Paclitaxel. Despite the fact that numerous hurdles need to be overcome, application of fungal production of this important antitumor drug is a significant research theme. Studies in this field have generated fruitful results. A great number of Paclitaxel-producing fungi have been found from all over the world. Screening for more fungal isolates from diverse natural niches may facilitate to identify better start strains with higher yield of the compound. On the other hand, to illustrate the genetic basis of the biosynthesis of Paclitaxel in fungi should be put on the top of the working list. Revelation of Paclitaxel genetics will help to find out the mechanism of regulation of Paclitaxel biosynthesis and the exact reasons for its instability in the culture. Hopefully, bioinformatics tools and new genome sequencing techniques will certainly expedite the study toward the solution of existing difficulties.

Paclitaxel Production by Endophytes:

Endophytes are considered asymptomatic bacterial and fungal microorganisms that for at least a part of their life cycle inhabit the intercellular spaces in plant tissues. However, the original definition referred to any species living together, such as microorganisms found within a plant. Like many biological terms, this one is constantly evolving as our knowledge of plant micro biomes and host interactions increases. Recent evidence for bacterial endophytes within the cytoplasm and the periplasm suggests that the specification of intercellular spaces should be updated, at least for bacterial endophytes.

It has been predicted that at least one species is likely to be present within each of the 350,699 different plant species on Earth. Endophytic fungi cause symptomless infections but are closely related to bio trophic and necrotrophic pathogens. Indeed, some species appear to have swapped roles multiple times during their evolutionary history. Interestingly, infected plants acquire selectable advantages resulting from endophyte colonization, including improved growth and increased resilience to abiotic and biotic stress.

Over the last few decades, several genera of endophytic fungi have been shown to synthesize a plethora of valuable natural products. The pool of secondary metabolites produced by endophytic fungi is vast and includes phenolic acids such as tyrosol and p-coumaric acids, quinones, hundreds of terpenoids, plant hormones such as gibberellins and indole acetic acid, as well as antimicrobial compounds such as Hsp90 inhibitors. Endophytes have also been proposed to synthesize the potent cytotoxic compound Paclitaxel. The finding that *Taxomyces andreanae* isolated from the phloem of *Taxus brevifolia* was capable of Paclitaxel production caused an explosion of subsequent publications reporting similar capabilities in other endophytic microorganisms. Approximately 200 different fungi representing diverse orders are thought to produce Paclitaxel. Reported productivity has ranged from as little as 0.001 ng mL−1 to ~800 ng mL−1 in various isolates, but so far these results have not been independently reproduced. Therefore, it is still unclear whether some of these species can actually produce taxanes, or whether the detected compounds were more likely artefacts of laboratory culture methods, or misidentified by the analytical and immunological methods deployed in the original studies. The most intriguing aspect is how so many fungi could acquire the independent ability to synthesize this complex diterpenoids when at least 19 enzymatic steps are required, and which other compounds they can produce. In many of the early publications claiming Paclitaxel production from isolated endophytes, the compound was detected using a competitive inhibition enzyme immunoassay. Without the necessary positive and negative controls, this method lacks stringency when only ng levels of a target compound are present.

Materials and methods

Chemicals and molecular reagents all the reagents and chemicals were of Analytical Grade Standard paclitaxel was procured from China. Microbiological media used for the growth and maintenance of Paclitaxel producing endophytes & electrophoresis reagents, molecular grade chemicals used for DNA isolation were purchased from Hi-Media & SD fine Chemicals. Coated Silica gel TLC plates, and HPLC solvents were procured from Merck (Germany) & Sigma (USA). Universal primers and Plasmids were procured from Thermo Fischer & Sigma Aldrich.

Collection of plant samples, isolation of Paclitaxel producing endophytes & Cell Line development of Taxus Spp.

Paclitaxel producing endophytes were isolated from different plant tissues (bark, stem, and needle) of Taxus sp. collected from Bhattu Buhla, Baijnath Tehsil in Kangra District Himachal Pradesh (India). The location map of the plant tissue collection sites is shown in image. The bark, stem, and needle samples were surface sterilized under a laminar airflow chamber with ethanol (70 %; v/v) for 30 sec. and sodium hypochlorite (3.5 %; v/ v) for 2 min, followed by washing with sterile water and similarly outer surface was peeled off using a sterile surgical blade. The bark, stem, and needles were cut into small pieces of 1 cm and were aseptically transfer on the surface of Mycological agar with composition of Dextrose monohydrate 50 g/L, Soya Peptone 20 g/L, Sodium acetate 2 g/L, Sodium benzoate 100 mg/L, Agar 30 g/L, pH 6.0–6.5 and were incubated at 25°C for 120 hrs. Morphologically different colonies were picked up depending upon shape, size, and colour. Pure cultures and Cell Line were established Picking & streaking single colonies on the PDA (Potato Dextrose Agar), SDA (Sabouraud Dextrose Agar & MMA (Modified Mycological Agar). Pure cultures were maintained on Glycerol Stock at -20°C. Same Plant samples cut into very small pieces surface sterilized under a laminar airflow chamber with ethanol (70 %; v/v) for 30 sec. and sodium hypochlorite (3.5 %; v/ v) for 2 min, followed by washing with sterile water and transfer into Murashige and Skoog Agar & Medium with Hormone and vitamin supplements for cell line development through tissue culture. Tissue culture involves the use of small pieces of plant tissue (**explants**) which are cultured in a nutrient medium under sterile conditions. Using the appropriate growing conditions for each explant type, plants can be induced to rapidly produce new shoots, Roots & Callus with the addition of suitable hormones.

Molecular screening of Paclitaxel producing endophytic Fungi.

All the isolated fungal cultures were grown individually in Erlenmeyer flasks containing 50 mL Modified fungal and Mycological liquid broth, consisted of glucose 6 g/L, sodium acetate 2 g/L, sucrose 36 g/L, Yeast-extract 10 g/L, fructose 18 g/L, soy Peptone 10 g/L, thiamine 2 mg/L, biotin 2 mg/L, pyridoxal 2 mg/L, calcium pantothenate 2 mg/L, magnesium sulphate 7.2 mg/L, calcium nitrate 13 mg/L, copper nitrate 2 mg/L, zinc sulphate 5 mg/L, manganese chloride 10 mg/L, iron (III) chloride 4 mg/L, phenylalanine 10 mg/L, sodium benzoate 200 mg/L, BAP (benzyl amino purine) 1 mg/L and NAA (naphthalene acetic acid) 1 mg/L pH 6.0–6.5 and 2 mL of 1 M sodium phosphate buffer, pH 6.8 and incubated at 25 °C for 5–7 days at 100 rpm in an incubator shaker.

Fungal DNA extraction :

Presence of tough chitinous cell wall in fungi is a challenge for cell lysis and subsequent DNA isolation which has several downstream applications. Genomic DNA was extracted using the CTAB method with necessary modifications and the procedure used was based on sodium dodecyl sulphate/phenol/chloroform extraction method to remove polysaccharides and protein. The isolated genomic DNA was used to successfully amplify ITS region. The amplified ITS region was sequenced and homology of its sequence was used for confirming identity of the fungal endophyte. This rapid and inexpensive method can efficiently isolate high quality and quantity of genomic DNA suitable for PCR amplification and sequencing. Briefly, the mycelia collected from the cultures were crushed in liquid nitrogen into a fine powder. 200 mg of mycelium powder was suspended in 1000 mL of DNA extraction buffer. To the above suspension, 200 mL of 5 M NaCl and 100 mL of 10 % Cetyl tri methyl ammonium bromide (CTAB) were added. The resulting mixture was incubated in a water bath for 3 min at 45°C with occasional inversion. An equal volume of Chloroform: Iso amyl alcohol (24:1; v/v) was added to the lysed mixture and centrifuged at 12,000 RPM for 30 min. The upper aqueous layer was collected gently and incubated with an equal amount of isopropanol overnight at 20 °C for precipitation. Post incubation, tubes were centrifuged at 25,000 RPM for 10 min (4°C), followed by washing of upper aqueous phase with 70 % ethanol. Finally, the pellet was dissolved in mili-Q (Nucleotide free) water (30 mL) and stored at 20°C till further use.

Nucleotide sequencing and phylogenetic analysis.

Specific bands of ITS and dbat genes were sliced from the agarose gel and purified by Thermo Scientific Gene JET Gel Extraction Kit. Sequencing PCR of the purified product was done by 785001KT Thermo Sequenase™ Cycle Sequencing Kit. Using appropriate sense and antisense primers for ITS and dbat gene with standard reaction and temperature profile. Specific amplified products were precipitated, and finally, the

samples were loaded into SeqStudio™ Genetic Analyzer System with Smart Start Series 3500 XL (Applied Biosystem, USA). Sequence analysis and comparison using the Basic Local Alignment Search Tool (BLAST) multiple sequence alignment and phylogenetic tree analysis were done using Clustal X 2.0 and Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (www.megasoftware.net) software respectively, based on the internal transcribed spacer sequences of similar fungal species.

Aligned Sequence Data of Sample – AFI-014 (1729 bp) Aspergillus Fumigates.

> AFI-014

AACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCATGTCTAAGTATAAGCAATTTATA
CGGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTTACTACATGGATACCTGTGGTAAT
TCTAGAGCTAATACATGCTAAAAACCTCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGG
GCTCCTTGGTGAATCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTAT
CAACTTTCGATGGTAGGATAGTGGCCTACCATGGTGGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGG
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GACAATAAATACTGATACGGGGCTCTTTTGGGTCTCGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGAAC
AATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAA
AAAGCTCGTAGTTGAACCTTGGGTCTGGCTGGCCGGTCCGCCTCACCGCGAGTACTGGTCCGGCTGGACCTTTCCTTC
TGGGGAACCTCATGGCCTTCACTGGCTGTGGGGGGAACCAGGACTTTTACTGTGAAAAAATTAGAGTGTTCAAAGCA
GGCCTTTGCTCGAATACATTAGCATGGAATAATAGAATAGGACGTGCGGTTCTATTTTGTTGGTTTCTAGGACCGCCG
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CGTCGTAGTCTTAACCATAAACTATGCCGACTAGGGATCGGGCGGTGTTTCTATGATGACCCGCTCGGCACCTTACGA
GAAATCAAAGTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGGAAGGGCACCA
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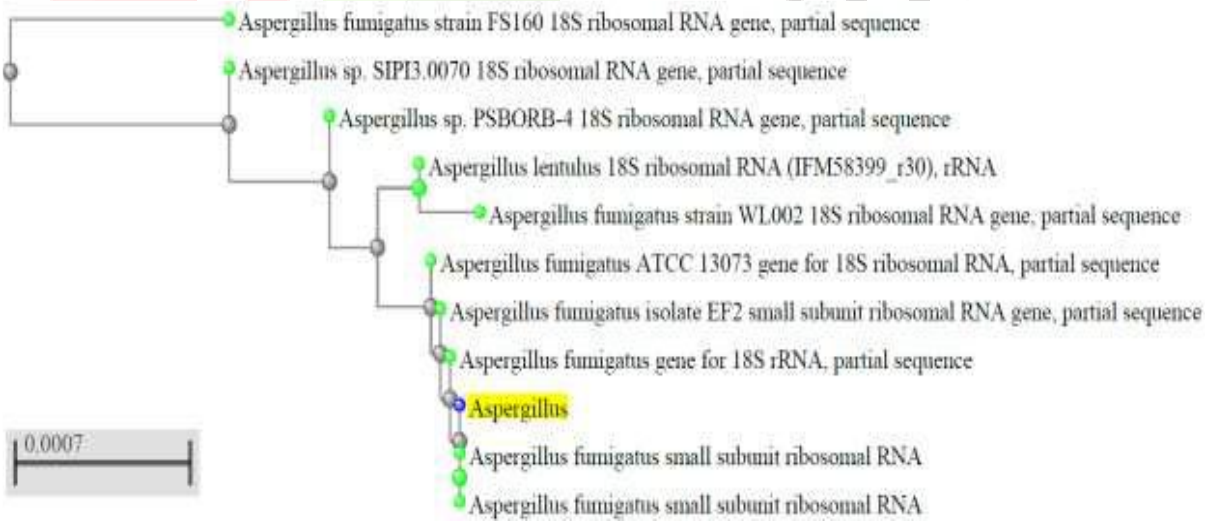
GCGATAACGAACGAGACGATGGAAGTGCGCGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCG
CGCTACACTGACAGGGCCAGCGAGTACATCACCTTGGCCGAGAGGTCTGGGTAATCTTGTTAAACCCTGTCGTGCTG
GGGATAGAGCATTGCAATTATTGCTCTTCAACGAGGAATGCCTAGTAGGCACGAGTCATCAGCTCGTGCCGATTACG
TCCCTGCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGCTCGGTGAGGCCTTCGGACTGGCTCAGGGGAG
TTGGCAACGACTCCCCAGAGCCGGAAAGTTGGTCAAACCCGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTC
CGTAGGTGAACCTGCAGAAGGATCAAGC

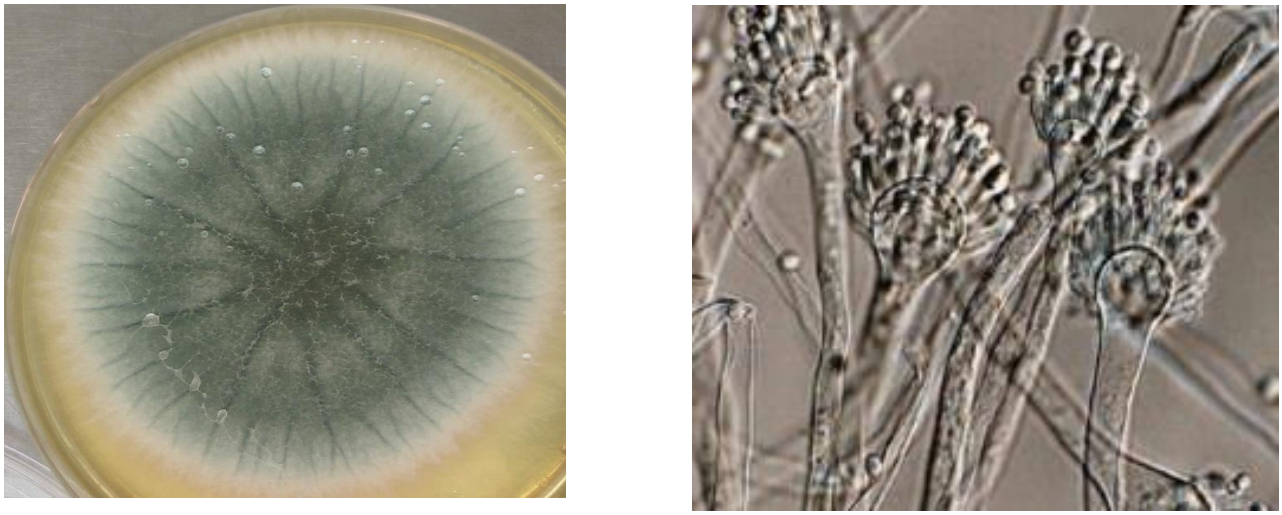
Identification and characterization of Paclitaxel producing endophytic fungus.

Fungal isolate AFI-014 found positive in molecular screening based on ITS gene expression, was further characterized using microscopic and molecular tools. The endophytic fungal strain AFI-014 was grown on 90 mm Petri plates containing MMA medium and characterized based on colony morphology, spores, reproductive structures, and 18S rRNA sequences.



Morphological characterization using microscopy.

Microscopic studies were carried out using fungal mycelia on a glass slide stained with Fungal Stain Lacto phenol Cotton Blue (LPCB) dye. Fungal mycelia were carefully spread using a needle, and a coverslip were placed onto the Microscopic Slide. Morphology was observed under the Biological microscope at 10 X, 40X & 100 X (Olympus, Japan).



Morphological characterization of Aspergillus fumigatus (a) on Modified Mycological Agar Medium (b) under Biological Microscope at 100X Without Staining

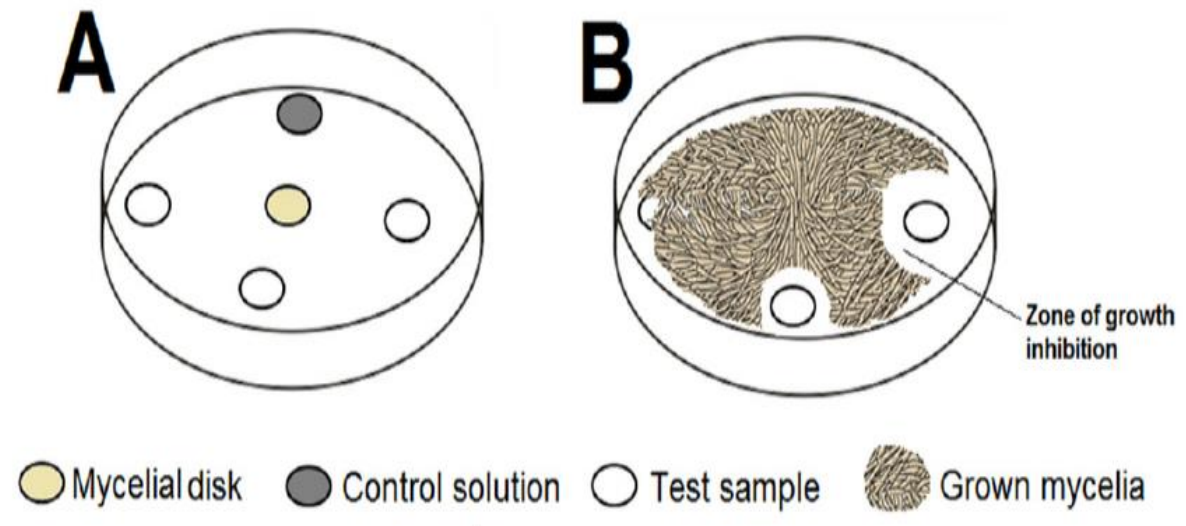
Antifungal bioassay:

The antifungal assay was performed on a PDA agar medium in Petri dishes using the dual culture method: - Endophytic fungi AFI-014 and two plant pathogenic test fungi, were cultured separately on PDA medium at 25°C for 5–7 days. The mycelial plugs (5 mm in diameter) of pathogens and endophytic fungi were placed on the same Petri dish 6 cm one from another. Dishes inoculated only with test pathogens were used as controls. Plates were incubated at 25°C for 5-7 days. The experiment was performed in triplicate. The percent growth inhibition of fungal pathogens was calculated by the following formula:

Percent inhibition = Radius of the pathogen in the control plate - Radius of pathogen in the dual culture plate Radius of the pathogen in the control plate x100

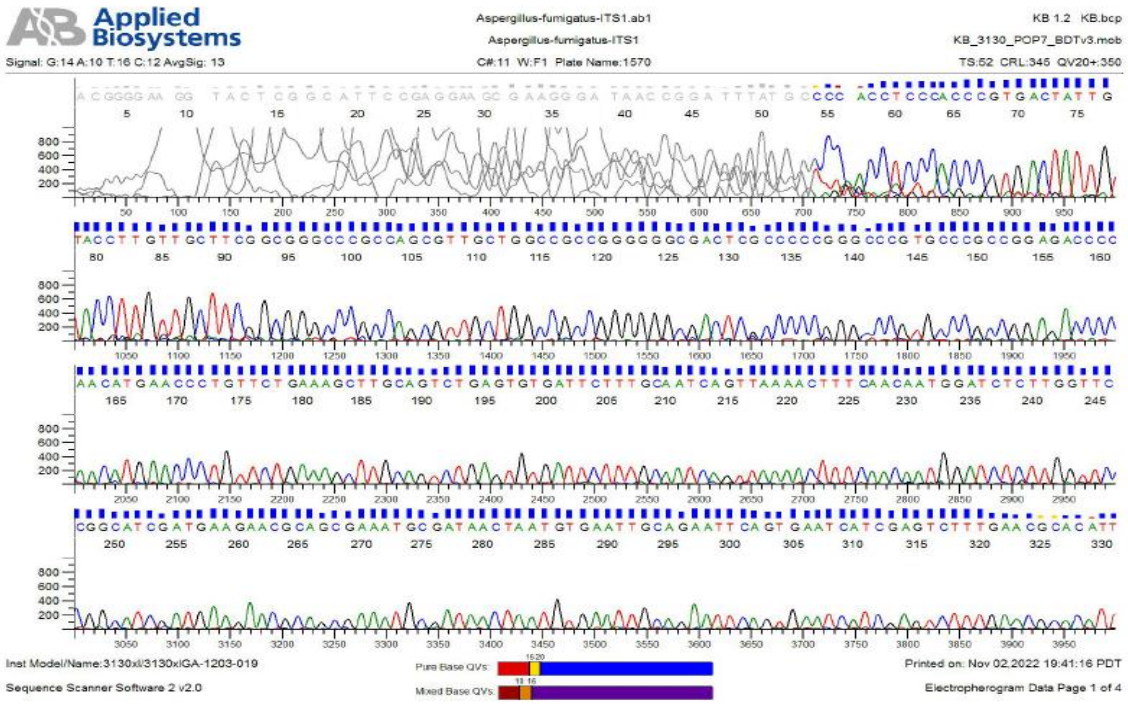
The percentage of inhibition was categorized on a growth inhibition category scale from 0 to 4 where
1. 0–30% growth inhibition means low antifungal activity
2. 31–50% growth inhibition means moderate antifungal activity
3. 51–70% growth inhibition means high antifungal activity
4. 71–100% growth inhibition means very high antifungal activity

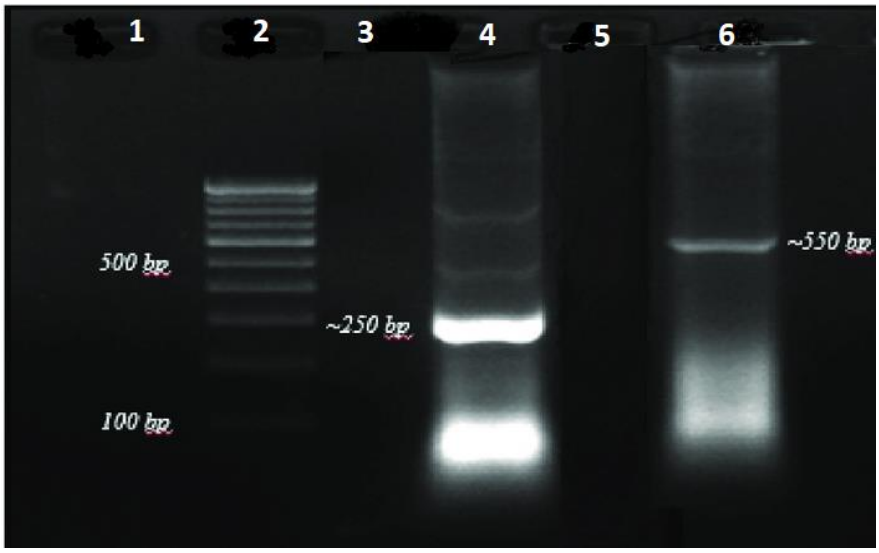
The endophytic fungi with a percentage of inhibition of more than 30% were kept for further study. Endophytes showing a good or moderate antifungal activity were selected for a secondary screening step, which was performed using the crown gall tumor disc method.



PCR based molecular screening using dbat and ITS genes

The internal transcribed spacer (ITS) fragments and 10- deacetyl baccatin III-10-O-acetly transferase (dbat) gene were amplified by using universal primers. ITS1 (5′-TCCGTAGGT- GAACCTGCGG-3′); ITS4 (5′-TCCTCCGCTTATTGATATGC-3′); dbat F (5′-GGGAGGGTGCTCTGTTTG-3′) and dbat R (5′-GTTACCTGAAC- CACCAGAGG-3′) were purchased from Sigma Aldrich (USA) & Thermo Scientific respectively. The standard PCR reaction of 25 mL consisted of 3 mL genomic DNA (100 ng), 1.5 mL forward and reverse primers each (10 mM), 0.4 mL DNA Taq polymerase (2 U), 2.5 mL 10X Taq buffer (Thermo), 2.5 mL MgCl2 (25 mM), 2.5 mL dNTP mix (2 mM), and 11.1 mL nuclease-free water (Thermo). The PCR reaction was performed by initial denaturation at 94 _C (3 min), followed by 30 cycles at 94° C (30 s), 55 °C (30 s), 72 °C (1 min) and final extension at 72 °C (5 min) using thermo cycler S1000 Thermal Cycler with 96-Well Fast Reaction Module 1852196. Besides this, taxadiene synthase (ts) and C-13 phenyl propanoid side chain-CoA acyl-transferase (bapt) genes involved in the Paclitaxel synthesis pathway were also screened for PCR amplification using primer sets for ts and bapt. The reaction and temperature profile for ts and bapt gene were similar for ITS gene, as mentioned above. Finally, the PCR products of ITS and dbat genes were analysed in 2 % agarose gel and visualized using Gel Doc system GS-900 Calibrated Densitometer by BIO RAD.





PCR analysis for the presence of dbat gene in Aspergillus fumigatus; Lane 2: Molecular Marker (100-1000 bp ladder); Lane 4: dbat gene (250 bp); Lane 6: ITS gene (550 bp); Lane 1, 3, 5: Empty.

Production and extraction of Paclitaxel from endophytic fungus

Erlenmeyer flask containing 50 mL Modified Paclitaxel Producing liquid medium (4.6±.2 pH) was Inoculate with spores of the fungal isolate AFI-14 and incubated at 25° C for 25 days with agitation speed at 100 rpm in an incubator shaker. Post 25 days of incubation, microbial biomass was removed from fungal isolates by passing the cultures through Whatman filter Paper. The fatty acid concentration was minimized by the addition of 0.25 g sodium carbonate to culture filtrate and later extracted with three equal volumes of ethanol and Ethyl Acetate. Under reduced pressure at 40°C (vacuum evaporator), the solvent was removed, leaving behind dry solid residues which were re-dissolved in methanol. The crude extract containing Paclitaxel was subjected to TLC, HPLC, UV-spectroscopy, FTIR spectroscopy, MS, and NMR analysis for the presence, quantity, and purity of Paclitaxel by comparing with standard Paclitaxel procured from China.

Characterization and analysis of extracted Paclitaxel.

Thin Layer Chromatography (TLC)

For the detection of Paclitaxel, the crude sample was spotted on 0.25 mm (10-20 cm) Aluminum pre-coated silica gel plates along with standard paclitaxel as internal standard, and the plate was developed in chloroform: methanol at 7:1 (v/v) successively. Paclitaxel was detected by spraying 1 % vanillin (w/v) in sulphuric acid after gentle heating or by using spray reagent consisting of 20 g of antimony trichloride in a mixture of 20 mL glacial acetic acid and 60 mL chloroform. The Retention factor (Rf) value of sample was calculated according to the following equation from the chromatogram and compared with standard Paclitaxel.

Rf value = Distance moved by the compound / Distance moved by the solvent.

Ultraviolet (UV) spectroscopic analysis

The UV spectroscopy analysis of the crude extracted sample was performed by scraping off the area of silica TLC plate containing putative Paclitaxel at the appropriate Rf. After dissolution in methanol, the spectrum of crude Paclitaxel samples was plotted in Beckman DU- 40 spectrophotometer (USA) and quantified by comparing with that of the standard Paclitaxel.

Fourier-Transform Infrared (FTIR) spectroscopic analysis

The extracted sample (crude Paclitaxel) was mixed and grounded with potassium bromide (KBr, IR grade) in a 1:10 ratio and pressed in Manual FTIR KBr Hydraulic Pellet Press to form pellet disc using spectra pelletizer. FTIR of the crude Paclitaxel was recorded and compared to standard paclitaxel with 5500 Series Compact FTIR Spectrometer in transmittance mode with a higher solution and a wide scan range of 4000 cm–1 to 500 cm–1.

High-Performance Liquid Chromatography (HPLC) analysis

HPLC was performed to estimate Paclitaxel production in the sample extracts. For HPLC analysis, the sample extracts were diluted in the mobile phase and HPLC performed using 1200 series Agilent HPLC equipped with reverse phase C18 5 mm Kromasil column (Sigma Aldrich) with UV, VIS, PDA Absorbance Detector. Extracted test samples (crude Paclitaxel) were filtered through a 0.2 mm filter. The mobile phase consisted of methanol: water, 80:20 (v/v). 10 mL of the crude sample was injected each time with 1 mL per min flow rate and was detected at 227 nm. Paclitaxel presence was verified by comparing the retention time of the test samples with that of the standard Paclitaxel (Paclitaxel, China).

Discussion:

Through this study of paclitaxel producing endophytes, their identification, Fermentation and extracted we concluded with crude powder. For confirmation of that powder as Paclitaxel we performed Characterization and analysis of extracted Paclitaxel powder like TLC, UV-Vis spectrophotometry, FTIR, HPLC and we received very positive results in form of confirmatory TLC spots, UV absorbance spectra, FTIR absorbance spectra chart and HPLC chromatograms with similar peaks on same RT & RRT. There are opportunities to discover novel fungal strains with interesting secondary metabolite production profiles because of the wide biodiversity of fungi in different plant types. Some tree species are likely to be hosts for hundreds of

endophyte species in their tissues. The ability to produce the same or similar host plant compounds was hypothesised the horizontal gene transfer of plant DNA into fungal genomes (Stierle et al., 1993). Recently, Xiong et al. (2013) reported a low similarity (<50%) of 2 key genes for Paclitaxel production pathway between plant and Paclitaxel-producing endophytes. They suggested that the ability of such endophytes might be involved from the initial coexistence between plants and endophytes during evolution. Thus, the horizontal gene transfer hypothesis should be further investigated where more key genes for Paclitaxel biosynthesis have been suggested to be involved in both plants and these endophytes. Many fungal endophytes have been found into classes of Ascomycota (Huang et al., 2001; Rodriguez et al., 2009; Tenguria et al., 2011 Although cell and tissue culture provides a potentially reliable and sustainable production platform, it is difficult to maintain high rates of secondary metabolite biosynthesis, The yield of secondary metabolites in cell suspension cultures must typically increase by several orders of magnitude before it reaches commercially acceptable levels, and this often requires the simultaneous use of many yield improvement strategies such as the manipulation of biotic and abiotic stimuli, scale-up techniques and the optimization of downstream processing.

Conclusion:

Synthetic biology has driven major advances in the development of microbial cell factories that produce natural products since the beginning of the 2000s. A few recent works have demonstrated the feasibility of production platforms for several families of plant-derived anticancer drugs – that is, opioids, cannabinoids, and alkaloids – and these successes suggest that we can anticipate the same for taxanes. As the plant producing paclitaxel has become endangered, the endophytes can be used as an alternative source for paclitaxel production. The endophytic fungus of the genus Aspergillus fumigatus. Its presence is confirmed in biological research as well as in a chromatographic and spectrophotometric analysis. The isolated fungi can be a good source of paclitaxel for large scale production by pharmaceutical industries in near future.

Species of the genus Aspergillus have been of great important fungi because of their beneficial and detrimental effects on human. Species of Aspergillus can produce various enzymes and organic acids, considering as cell factories. These fungi can produce many secondary metabolites, so it can be utilized as host species for generating novel metabolites that can be used for pharmaceutical purposes. Recent studies reported that species of Aspergillus can decompose plastics and pollutants, so these fungi can be utilized for the environmental field. Furthermore, through the development of genetic engineering and systemic biology, many researchers sought to use filamentous fungi as cell factories. Unlike bacteria or yeast, the available gene editing methods and transformation efficiency are low, but filamentous fungi are still attractive cell factories for industry. To develop Aspergillus as cell factories, currently, PMT and AMT-mediated gene targeting are mainly used in genetic engineering.

The productivity of Paclitaxel was enhanced by several orders of magnitude through a combination of genetic and environmental approaches. The future outcome of this process is for large production of paclitaxel appears to be quite robust. The plant kingdom is characterized by a tremendous biodiversity arising out of a large pool of genetic information which encodes for a broad spectrum of phytochemicals. Plant cell culture technology can be used effectively to tap into this chemical diversity, resulting in a large array of potentially useful pharmaceutical product leads. Application of this approach to the discovery of novel taxanes to be illustrate.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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