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## Full Length Research Paper

# A new taxol-producing fungus (*Pestalotiopsis malicola*) and evidence for taxol as a transient product in the culture

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**Fungal production of the anti-tumor taxol is an effective way of making this drug in industries. We reported here a new taxol-producing fungus, NK101, from plant debris in the soil. Based on the culture characteristics, conidia structure and molecular evidence, NK101 was classified as *Pestalotiopsis malicola*. Taxol was verified in both the culture and the mycelium in a high level (186 µg/L). The time course of yield suggests that taxol was present as a transient product in the fungus. This work may show the diversity of using fungi to produce taxol.**

**Key words:** Taxol, saprophyte, *Pestalotiopsis*.

## INTRODUCTION

Taxanes are a family of natural diterpene alkaloids that are originally found in the bark of the Pacific yew (*Taxus brevifolia*) (Yu et al., 2009; Wani et al., 1971). Paclitaxel (Taxol<sup>®</sup>) is the best known taxane member which has been used as an antitumor drug for the treatment of a broad array of cancers (Yu et al., 2009). The finding which shows that fungi are used to produce taxol and other taxanes has a great impact on the production of this drug (Ji et al., 2006; Stiele et al., 1993). Currently, taxol is being made by either direct extraction from yew tree or the conversion of baccatin III or 10-deacetyl baccatin III (10-DAB) to taxol via chemical synthesis. Industrial production cannot meet the increasing demand of the market due to the low availability of the precursors, baccatin III and 10-DAB that are still being extracted from the leaves and barks of yew tree (Cragg et al., 1993; Yu et al., 2009). Fungal fermentation for taxol and its precursors may pave an alternative way for solving the problem. Unfortunately, to a great extent, only a limited number of taxol-producing fungi have been found. The yield of taxol by fungi culture is generally low. Thus, searching for better taxol-producing microbes is still a

priority for scientists.

We reported here a new paclitaxel-producing fungus, NK101. Unlike the fungi reported before, we isolated this fungus from plant debris in the soil. Based on its culture characteristics, the conidia structure and the 18S rDNA sequence, it is identified as *Pestalotiopsis malicola*. This fungus produces abundant secondary metabolites. Serial biochemical analyses suggest the presence of taxol in the culture that is identical to the authentic taxol. As the standard, the purified taxol has a high toxicity to the testee oomycete *Pythium ultimum*.

## MATERIALS AND METHODS

### Fungal isolation and culture conditions

Briefly, the collected plant debris from Yunnan Province, China was washed with sterile ddH<sub>2</sub>O twice and was submerged in 70% ethanol for 5 min, and then placed on water agar containing 100 µl/ml ampicillin at 28°C for 5 days. The growing tips of hyphae were transferred to a fresh PDA plate (20% peeled and cut potato, 1.0% glucose and 1.5% agar, natural pH) at 28°C for 13 days to allow conidia formation. Individual conidia were isolated and plated on PDA to obtain pure fungal culture. The paclitaxel testee oomycete, *P. ultimum* was a courtesy gift from Dr. Binggan Lou (Zhejiang University, Hangzhou, China). PDA was also the medium to grow *P. ultimum*. PDB was the both of PDA without agar.

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### Extraction and characterization of paclitaxel from the culture of isolate NK101

Extraction and characterization of fungal paclitaxel was described by many authors (Stiele et al., 1993; Strobel et al., 1996). Briefly, fungal mycelium was collected from 200 ml PDB culture shaken at 180 rpm, 28°C for 6 days. The mycelium and the liquid were separated by vacuum filtration with filter paper. The mycelium was soaked in equal volume of methanol at room temperature for 2 days. The culture supernatant was extracted with equal volume of methanol twice. And the methanol extractions were concentrated by evaporation at 40°C to approximately 1.0 ml.

Thin layer chromatography (TLC) was adopted for primary screening. Extractions were spotted on GF254 silica gel plates and were developed in methylene chloride/ethyl acetate (6:1, v/v). Paclitaxel spots were detected under UV light at 235 nm which appeared to be bluish. The standard paclitaxel (Sigma, St Louis, USA) was run in parallel as control. The candidate spots were scraped off the silica plates and extracted with acetonitrile for further high performance liquid chromatography (HPLC) analysis. A Kromasil C<sub>18</sub> ODS column (4.6 × 250 mm, AKZO Nobel, Switzerland) was used in the HPLC (Agilent 1100, Agilent Technologies, CA, USA). Samples in 20 µl methanol were injected and then eluted with methanol/H<sub>2</sub>O (70:30, v/v; pH 7.0). The flow rate was 1 ml min<sup>-1</sup>. The standard paclitaxel served as the control. After purification, taxol from NK101 was further verified by a liquid chromatography-mass spectrometry (LC-MS) analysis facility (SHIMADZU LCMS-2010, Tokyo, Japan) (McClure et al., 1992; <https://cp.chem.agilent.com/Library/applications/5967-5901.PDF>). The sample in methanol/water/acetic acid (70:30:1 by volume) was injected with a spray flow of 0.3 ml min<sup>-1</sup> in C-18 column (Agela Technologies, Vensil XBP C-18, 2.5 µm, 100 Å, 3.0 × 75 mm), detected under 227 nm ultraviolet and a spray voltage of 0.1 kV by the loop injection. Nuclear magnetic resonance (NMR) was also employed for fungal samples. NMR was carried out with a Bruker 600 MHz instrument with the sample dissolved in 100% deuterated chloroform. The sample was subjected to 2048 scans with a sweep width of 6024 and 8 K real points.

### Sequence determination of 18S rDNA of NK101

Genomic DNA was prepared for PCR amplification of the fungal 18S rDNA. Strain NK101 was grown in 200 ml PDB at 28°C for 10 days. The mycelium was lyophilized and ground. A universal pair of primers for the amplification of fungal 18S rDNA, UK4F (5'-CYGGTTGATCCTGCCRG-3') and UREV (5'-GYTACCTTGTACGACTT-3') were used (Metz et al., 2000). To amplify the ITS1-5.8S rRNA-ITS2 region, the universal primers, ITS1/ITS4, were used (5-TCCGTAGGTGAACCTGCGG-3/5-TCCTCCGCTTATTGATATGC-3). The PCR products were gel-purified with the BioSpin Gel Extraction Kit (Bioer Technology Co., Hangzhou, China). The PCR products were sequenced and deposited in GenBank (Accession No. HM130668 and JF501649, respectively).

### Anti-oomycete activity of the fungal taxol

For further confirmation, purified taxol was tested for anti-oomycete activity with a testee strain *P. ultimum* that is sensitive to paclitaxel (Mu et al., 1999; Yong et al., 1992). A simplified inhibition zone assay was designed for the test (Figure 4). The standard taxol from Sigma served as control. 50 µl of each sample (10 µM in methanol) was added to an autoclaved steel test cup that was placed on the PDA plate. A slice of agar carrying *P. ultimum* mycelium was inoculated at the center of the plate at 22°C for 3 days. The inhibitory effect of paclitaxel on *P. ultimum* was manifested by the

formation of an inhibition zone.

### Taxol production versus culture time in NK101

To observe the kinetic status of paclitaxel biosynthesis in the culture, quantity of paclitaxel was determined by comparing the area of the fungal paclitaxel peak on HPLC spectrum to a standard curve made with the standard sample (Strobel et al., 1996). 10<sup>6</sup> conidia were inoculated to 200 ml PDB and grown as described earlier in triplicate. The fungal paclitaxel was prepared at various time points. And a curve of the quantity of produced paclitaxel versus culture time was created.

## RESULTS AND DISCUSSION

### NK101 is *P. malicola* Hori and a diversity of natural niches for taxol-producing fungi

Based on the culture characteristics and the reproductive structure of the fungus, NK101 is classified as *P. malicola* that has been described in China by Ge et al. (2009). Conidiomata are black, acervular, scattered and amphigenous at early stage. Mycelium is thin, yellowish to brown and has no growth rings (Figure 1, left panel). Conidia have five cells with four septa, coryneform, erect or curved, constricted at septa, double pigmented septa and 20.6 × 5.1 µm in size. Both the basal and apical cells are hyaline, and the median three cells are dark brown. The conidia usually have 3 apical setulae with an average length of 22.4 µm (Figure 1, right panel). Two or four setulae were also observed. The setulae are usually longer than the conidia. A short basal pedicel is measured to an average length of 3.44 µm. The 1768-bp 18S rDNA gene from NK101 shared a 99.0% identity to that of *Pestalotiopsis microspora*. Also, the ITS1-5.8S rDNA-ITS2 has a 99.0% identity to that of several species from the genus *Pestalotiopsis*, suggesting that this fungus belong to the genus *Pestalotiopsis* (>97%). Unfortunately, no sequences of 18S rDNA or ITSs of *P. malicola* have been found in the GenBank. However, the conventional taxonomic criteria based on the overall morphology, culture characteristics and conidia structure, in combination with the sequence data support the fact that NK101 is an isolate of *P. malicola* (Strobel et al., 1996). In fact, a taxol-producing *P. microspora* strain 07c that is easily distinguished from *P. malicola* was also isolated by us (unpublished data).

Based on the information we collected, approximately twenty (20) different species have been reported on international journals since the first taxol-producing fungus was found in 1993 (Stierle et al., 1993). Taking a close look, all these fungi are exclusively plant endophytes. It seems that people have only been focusing on plant endophytes for taxol-producing fungi. This may limit the finding of more taxol-producing fungi. We have started to examine fungi from more plant hosts other than yew tree and environmental substrates. We have obtained some taxol-producing fungi from soil from



**Figure 1.** Growth of NK101 on PDA (left panel) and its conidia (right panel).

the south provinces of China including *P. malicola* NK101. This alteration of isolation strategy may increase the odds to get better fungal strains for industrial application. It is also worthwhile to note that among the identified taxol-producing fungi, more than one-third (8/20) are from the genus *Pestalotiopsis* (*P. microspora*, *Pestalotiopsis guepin*, *Pestalotiopsis versicolor*, *Pestalotiopsis neglecta*, *Pestalotiopsis breviseta*, *Pestalotiopsis pauciseta*, *Pestalotiopsis terminaliae* and *Pestalotiopsis malicola*), strongly suggesting that these fungi may have a common taxol-producing ancestor. These fungi including *P. malicola* are widely distributed plant pathogens all over the globe (Ge et al., 2009). Whether taxol and related taxanes play a role in pathogenesis is an interesting question.

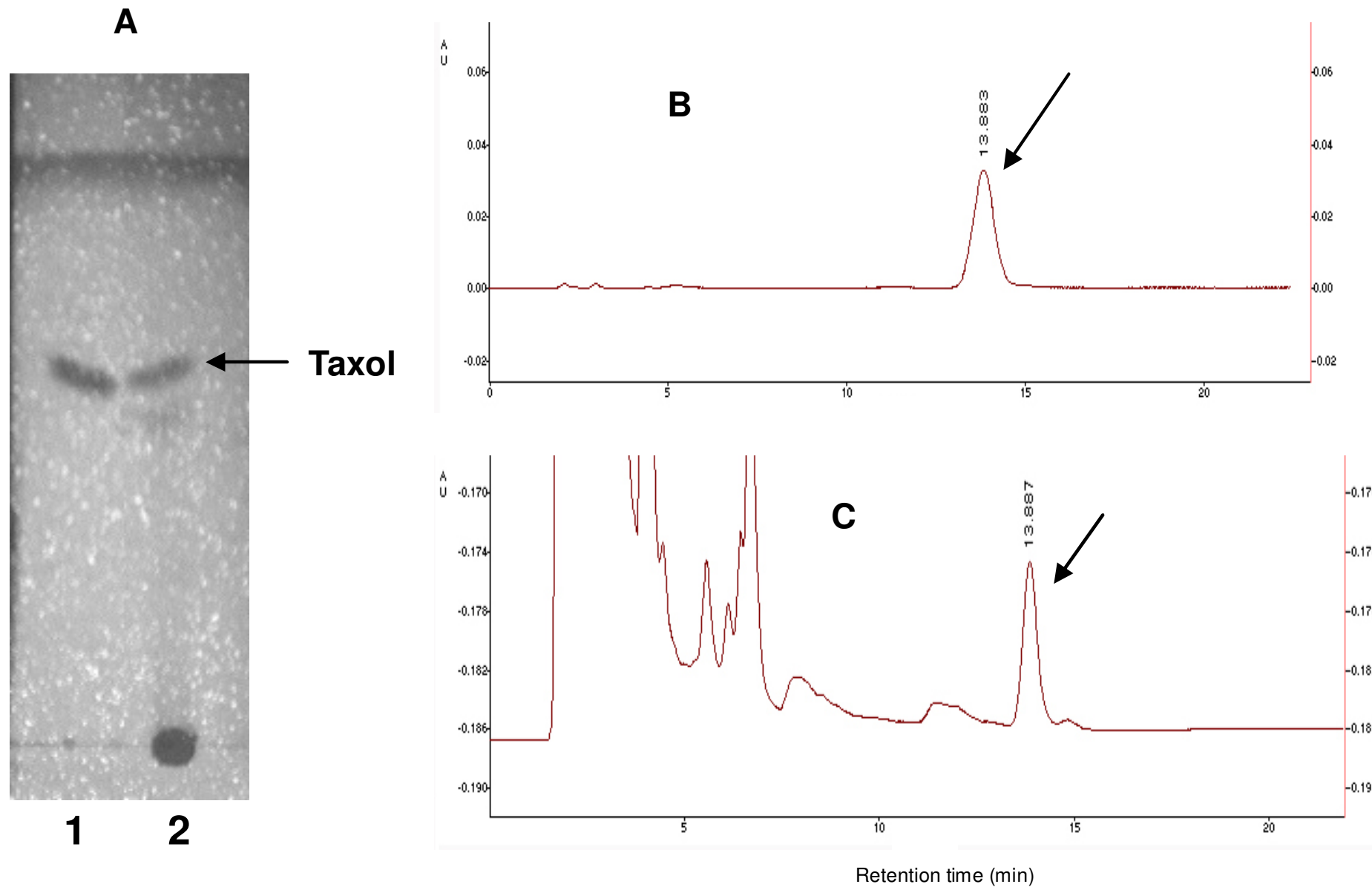
### Characterization of taxol from NK101

The extract from NK101 contained a compound that had a similar  $R_f$  value to the standard taxol that had an absorbance at 235 to 254 nm ultraviolet on TLC plates (Figure 2A). The retention time (13.887 min) of the putative fungal taxol (Figure 2C) was close to that (13.883 min) of the authentic sample from Sigma (St. Louis, USA) on the HPLC chromatogram (Figure 2B). The component at 13.887 min in HPLC was collected for further LC/MS and NMR analyses. LC-MS data confirmed a characteristic peak at 876.2 ( $M+Na$ )<sup>+</sup> for the fungal compound (Figure 3A) and a taxol peak at 854 ( $M+H$ )<sup>+</sup> (not shown). Besides, the fungal paclitaxel shared an identical NMR spectrum with that of authentic taxol (Figure 4). Taken together with the later biological assay, these results verified that NK101 produces the authentic taxol. As a matter of fact, characterization of a known compound like taxol has become straightforward with the advance of analytic tools, such as the high resolution

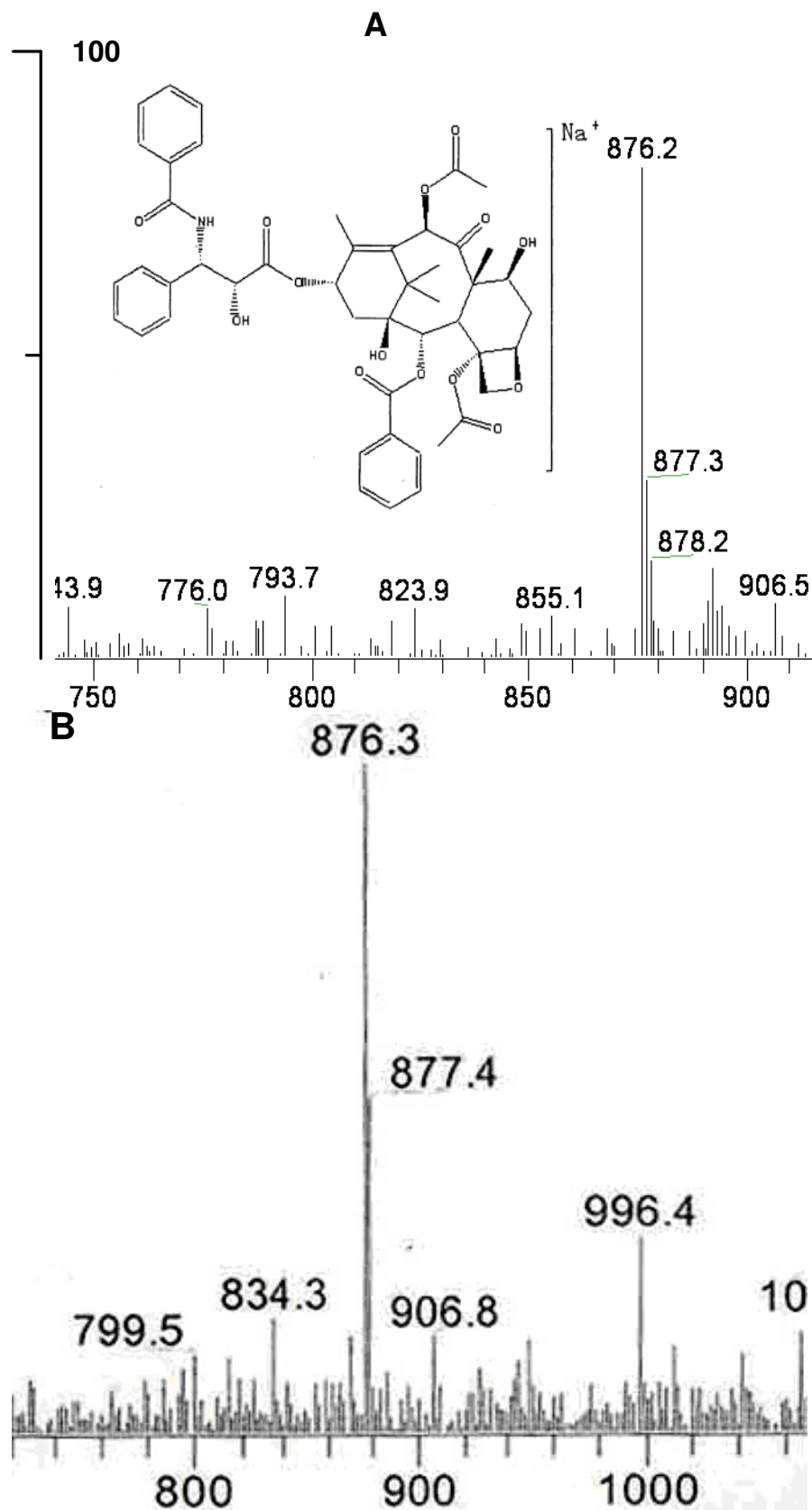
mass spectrometry and a variety of HPLC in conjunction with mass. With the LC/MS method, one is able to distinguish the two closest isomers such as paclitaxel (taxol) and 7-epipaclitaxel (Application Note 174, SUPELCO, PA, USA). Lately, few authors included only homologous gene sequences to support the view that fungi have the capability to make the chemical (Zhang et al., 2009). Considering the far evolutionary relationship between fungi and yew trees, the assumption that the two groups of organisms share the identical genetic basis for taxol biosynthesis might be still far-fetched. By far, none of the genetic components for taxol biosynthesis in fungi have been confirmed by disruption. Even in yew, cloned genes has not been decisively demonstrated by loss-of-function approaches with genetic manipulation.

### Anti-oomycete activity of NK101 taxol

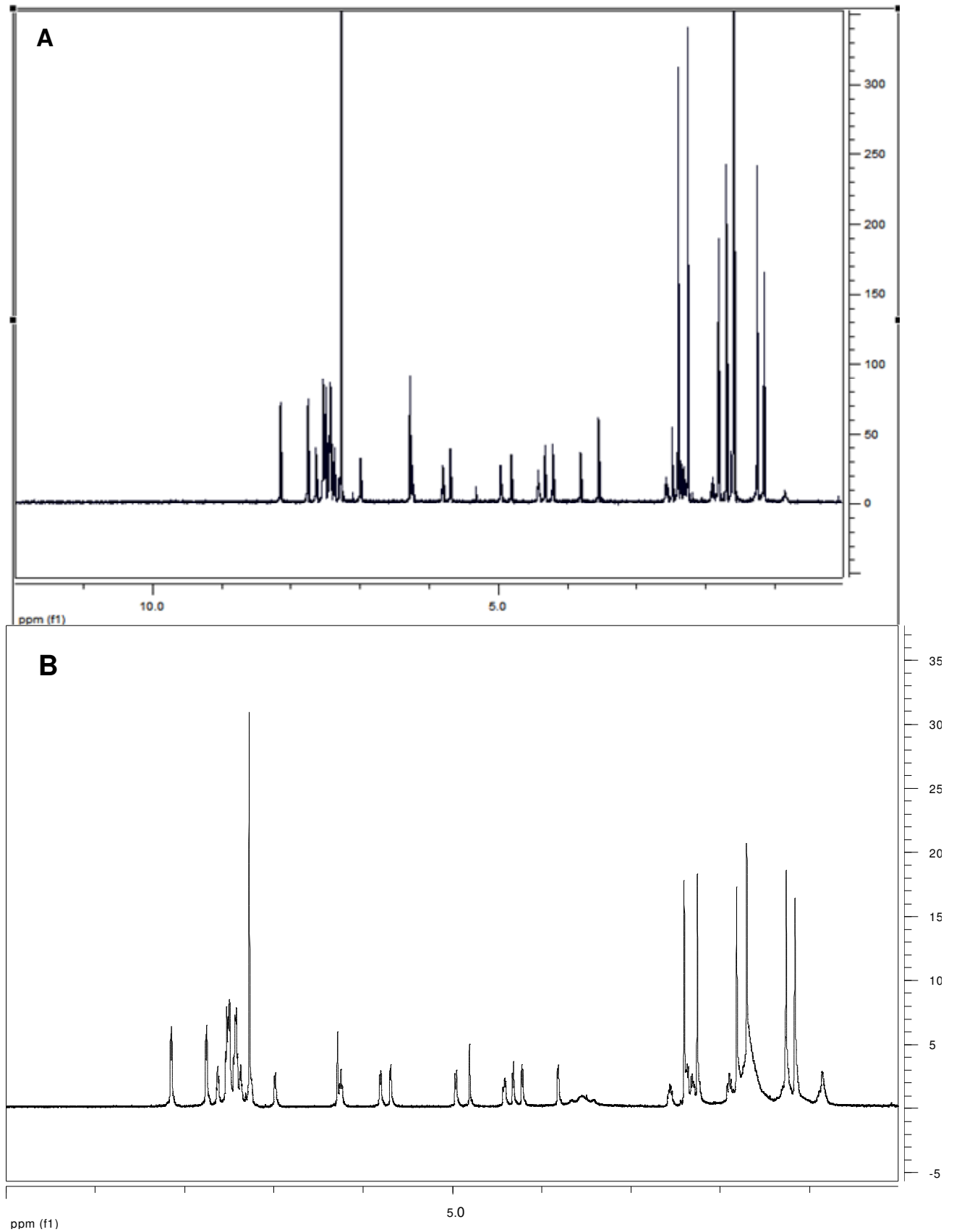
An inhibition assay of tumor cell lines is often included. This assay is tedious and needs expensive equipment. With more microbiological laboratories getting involved in the study of taxol, a simple test should be beneficial. Taxol is toxic to oomycetes by the same mode of action as that on tumor cells (Mu et al., 1999; Young et al., 1992). Thus, we designed a simplified assay with *P. ultimum* as a testee. 50  $\mu$ l of each taxol sample (10  $\mu$ M in methanol) was added to the test steel cups that were uprightly placed on a PDA plate (Figure 5). A small piece of agar containing *P. ultimum* was placed on the central spot of the plate to allow the mycelium to grow outward at 22°C for 48 h. The inhibition zones were formed around Cups A (fungal taxol) and B (the Sigma taxol control) due to the inhibited growth of *P. ultimum*, while no inhibition zone was seen around Cup C to which only methanol was added. This result clearly confirmed that the fungal taxol has the identical biological activity on *P. ultimum*.



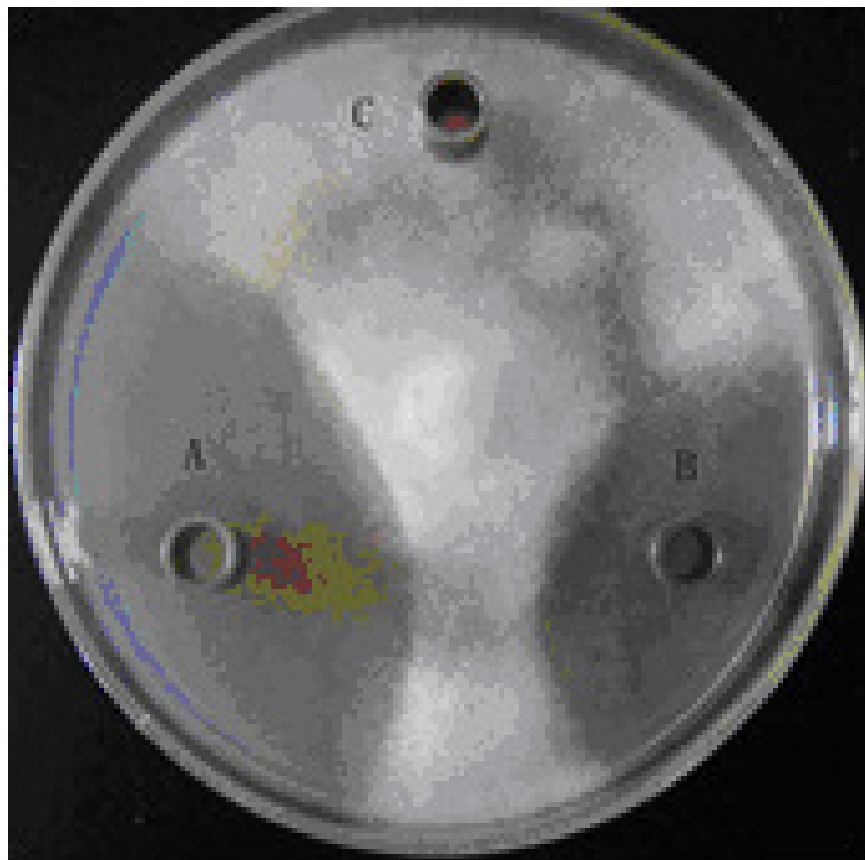
**Figure 2.** (A) The extract from NK101 (lane 2) contained a compound that had a similar Rf value with the standard taxol (lane 1) that had an absorbance at 235 to 254 nm ultraviolet on TLC plates. B and C: The retention time of the putative fungal taxol (C) was 13.887 min by the HPLC chromatogram. Similarly, the authentic taxol sample from Sigma (St. Louis, USA) had a retention time of 13.883 min (B).



**Figure 3.** Electrospray mass spectrum (EMS) of fungal taxol. The characteristic peak of taxol at 876.2 ( $\text{M}+\text{Na}^+$ ) was observed for the sample extracted from NK101 (panel A), close to characteristic peak at 876.3 for the standard taxol control (panel B).



**Figure 4.** NMR spectra of fungal and the standard sample. The spectrum of NK101 taxol (B) is identical to the standard sample (A).



**Figure 5.** Biological activity assay for the NK101 taxol. The oomycete *P. ultimum* was used as a testee. The mycelium of *P. ultimum* was plated on the center of the plate. Purified taxol in 50  $\mu$ l methanol (approximately 10  $\mu$ M) was added to cups. Inhibition zone was formed at 22°C for 48 h, by the fungal paclitaxel (A), and the standard sample at the same concentration (B); No inhibition zone was observed for the solvent methanol alone (C).

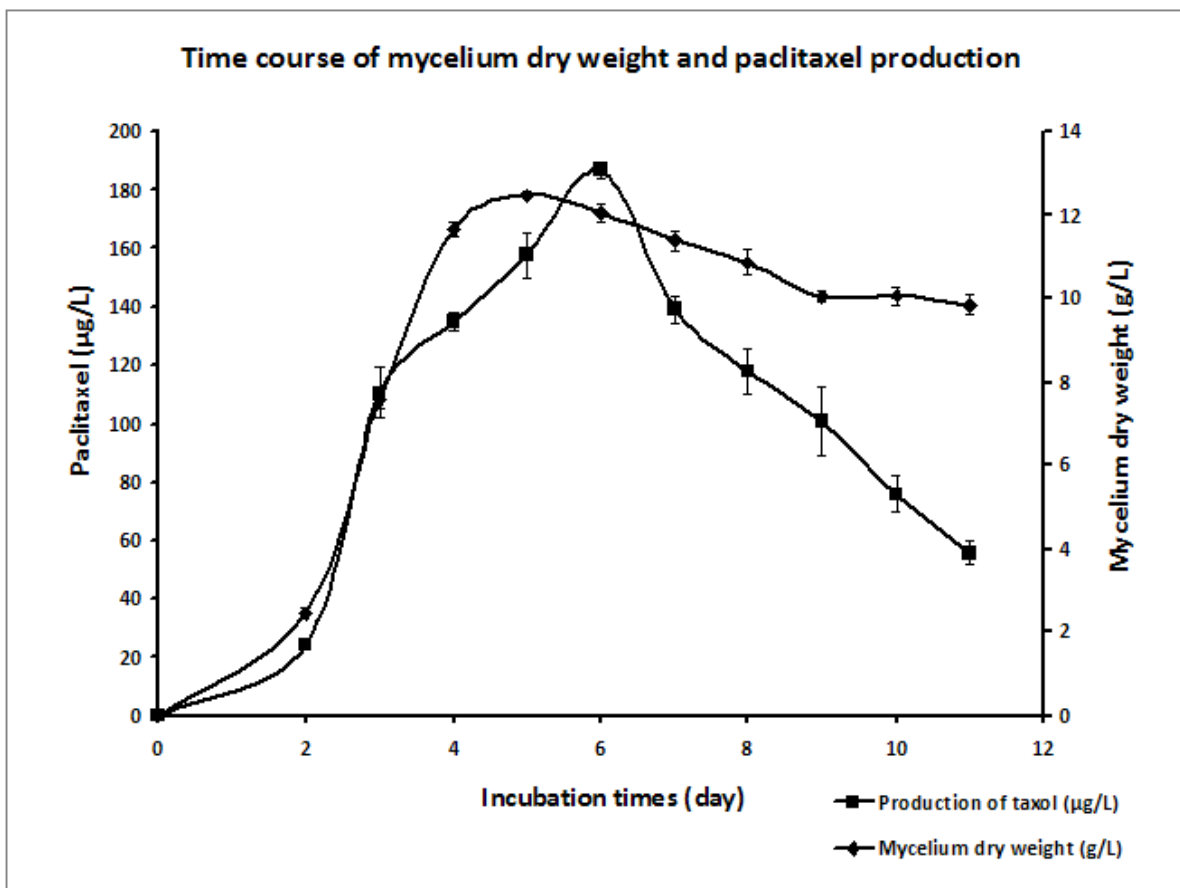
Beforehand, we determined the inhibition concentration of taxol on *P. ultimum*. As low as 0.1  $\mu$ M, taxol formed an apparent inhibition zone on the plates (unpublished data). Sensitivity of *P. ultimum* to taxol seems to be strain-specific. Another *P. ultimum* strain in our laboratory isolated from cabbage showed little sensitivity to taxol (unpublished data).

#### **A time course of taxol production in liquid culture of NK101 and its stability**

If a secondary metabolite is the end product of a metabolic pathway, it should be accumulated in the culture. To see whether taxol is accumulated by NK101, we determined total amount in both mycelium and the culture versus various time points (Figure 6).  $10^6$  conidia were inoculated to 200 ml PDB at 28°C and 180 rpm. The mycelium was harvested, dried and weighed as the indicated date. As shown in Figure 6, the growth of NK101 reached the maximal level on the fifth day. The

maximal taxol quantity was obtained on the sixth day and the yield reached 185.9  $\mu$ g/L and then declined rapidly to an undetectable level after 18 days (data not shown). This result is consistent with the early observation for another fungus *P. microspora* (Strobel et al., 1996). In that report, the maximum yield of taxol was reached after three weeks in a still culture and also showed fast declination afterward. Additionally, the yield per liter culture of our fungus is nearly 3-times higher than that of *P. microspora*. We found that approximately 60 to 70% taxol were excreted into the culture.

In fact, the time course provides a hint in respect to the status of taxol in fungi. We interpret this instability as a normal conversion to other substances or degradation may occur following taxol biosynthesis with time and culture conditions. In other words, fungal taxol stays as a transient product and undergoes further metabolism in the fungus. Theoretically, if the downstream pathways are defined and then blocked by genetic engineering strategy, taxol is expected to accumulate and the yield of taxol should increase. This goal is obviously more pragmatic to



**Figure 6.** A time course of taxol production in NK101 and the growth curve (dry mycelium weight). The taxol preparation and the quantity determination were described in the section of materials and methods. Total taxol from both the mycelium and the culture reached the highest level on the sixth day and fell sharply to undetectable level after 18 days. The growth reached its maximal level on the fifth day.  $10^6$  conidia were inoculated into 250 ml PDB, shaken at 180 rpm, 28 °C. The assay was conducted in triplicate and the error was expressed as standard deviation.

achieve in fungi than in yew tree.

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