

Full Length Research Paper

Investigation of fermentation conditions and optimization of medium for taxol production from taxol-producing fungi

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The monofactor-design test was used to optimize the culture conditions for the fermentation of taxol-producing fungus UV₄₀₋₁₉ as follows: the initial pH value of the fermentation broth was 5.5, the culture temperature was 25°C, and the rotational speed was 150 r/min. Orthogonal design was used to optimize the fermentation medium of taxol-producing fungi. The optimized medium increased the yield of taxol to 456.24 ± 2.83 µg/L, which is 115% of the yield obtained with the modified S-7 medium. The results showed a great potential for producing taxol on an industrial scale, which would lead to significant economic and social benefit in the future.

Key words: Taxol-producing fungi, taxol, fermentation, medium optimization.

INTRODUCTION

Taxol is a novel functional taxane diterpene amide with anti-tumor activities, firstly isolated from the bark of *Taxus brevifolia* (Wani et al., 1971). Clinically, taxol has been used successfully for the treatment of many malignant tumors, such as breast cancer, adenocarcinoma and squamous cell carcinoma of the esophagus (Woo et al., 1996; Jones et al., 1996; Pulkkinen et al., 1996). At present, taxol is still mainly extracted from the bark of yews. However, this method cannot meet the increasing demand for taxol on the market because yews grow very slowly and are a rare and endangered species belonging to first-level conservation plants in China. Recently, increasing efforts have been made to develop alternative means of taxol production, such as using complete chemical synthesis, semi-synthesis and the *Taxus* spp. plant cell culture. Using microbe fermentation in the production of taxol would be a very prospective method

for obtaining a large amount of taxol. In 1993, Strobel isolated an endofungus *taxomyces andreanae*, which can produce 24 to 50 ng/l taxol (Strobel et al., 1993). Thereafter, Qiu et al. (1994); Strobel et al. (1996) and Li et al. (1996) reported the isolation of taxol-producing endofungi from *Taxus* and other plants. The fungi exhibit a taxol yield of 95-1081 ng/l. Since 1993, the authors have isolated five new endophytic fungal species that produce taxol by screening samples from the inner bark (phloem-cambium) and xylem of *Taxus cuspidata* Sieb. and Zucc. These fungi are *Nodulisporium sylviforme* (Zhou et al., 2001), *Pleurocytospora taxi* (Sun et al., 2003), *Alternaria taxi* J.P. Ge et al. (Ge et al., 2004), *Botrytis* (Zhao et al., 2008a), and strain HD86-9 (Zhao et al., 2009). *N. sylviforme* The production of taxol by *N. sylviforme* was estimated to be between 51.06 and 125.70 µg/l. After a series of mutagenesis, strain NCEU-1 that produced 314.07 µg/l taxol was obtained after multiple steps of mutagenesis screening of the spores from *N. sylviforme* (Zhou et al., 2001). The protoplasts of NCEU-1 were mutagenized by UV irradiation. A strain UV₄₀₋₁₉ that produced 376.38 ± 8.41 µg/l taxol was used as the starting material (Zhao et al., 2005). In order to increase the synthetic capability of taxol, this paper investigated the

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Table 1. Factors versus levels for orthogonal experiment design.

Factors Levels	Acetic ammonium (mg/l)	Salicylic acid (mg/l)	Silver nitrite (mg/l)	Serine (mg/l)
1	0.7	70	1.5	85
2	0.8	80	1.7	100
3	0.9	90	1.9	115

optimization of the fermentation conditions and medium for taxol production from strain UV₄₀₋₁₉.

MATERIALS AND METHODS

Strain and media

Strain UV₄₀₋₁₉ with a taxol yield of 376.38 ± 8.41 µg/l was used as tested strain. Potato dextrose agar (PDA) liquid medium, PDA solid medium (PDA liquid medium containing 2% agar), modified S-7 medium was based on S-7 medium with the addition of tyrosine, linolitic acid and phenylalanine at the final concentrations of 1.5, 1.5 and 5.0 mg/L, respectively (Zhao et al., 2003). All media were autoclaved for 20 min prior to use.

Activation of strain UV₄₀₋₁₉

The strain UV₄₀₋₁₉ were spread onto PDA slant medium and cultured for 3 days at 28°C, and then activated in PDA liquid medium (50 ml PDA liquid medium in a 250 ml conical flask) for 3 days at 28°C, 120 r/min.

Fermentation test

The seeding culture fluid (mycelium) was seeded onto the modified S-7 medium (200 ml modified S-7 medium in a 500 ml conical flask) with an inoculating ratio of 3 to 97 and then cultured for 12 days at 28°C, 120 r/min in order to optimize the fermentation conditions.

Biomass quantification

From the second day, a certain amount of fermentation broth was taken out every day until 14 days after inoculation, followed by filtration using filter paper. The mycelium was washed twice by aquae sterilisata, then dried at 45°C and weighed.

Optimization of the medium compositions

Using L₉ (3)⁴ orthogonal design based on the optimal fermentation conditions determined above, the effects of different levels of precursors and inducers on the yield of taxol from the taxol-producing strain UV₄₀₋₁₉ was studied using the modified S-7 medium as controls (Table 1).

Extraction and purification of taxol

The extraction and purification of taxol was carried out as previously described (Zhao et al., 2011).

Mass spectrometry analysis

Mass spectrometry analysis was carried out as previously

described (Zhao et al., 2011).

Statistical analysis

All experiments were repeated five times and each measured in triplicate. The results were expressed as mean \pm SD. Mean values were analyzed using the double-sided Student's *t*-test. Differences were considered to be statistically significant with $p < 0.05$.

RESULTS AND DISCUSSION

Monofactor test for fermentation conditions

Initial pH

In this study, 3% (v/v) seeding culture liquid was inoculated into the modified S-7 medium with initial pH at 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, respectively. Then the cells were cultured at 28°C, 120 r/min for 12 days in shaking flasks. Figure 1 showed that the optimal pH is 5.5, at which the highest taxol concentration of 378.69 ± 1.71 µg/l was produced. In addition, the initial pH showed great effect on the yield of taxol. Therefore, it is important to control the initial pH value in the fermentation broth. It was reported that higher pH was helpful for growing *T. cuspidate* (Sheng et al., 2001). At pH 7.0, the wound tissue of *T. cuspidate* grows best; while at pH 6.0, the concentration of taxol is the highest. For *T. chinensis*, pH 5.5 is most suitable for the growth of wound tissue, and the concentration of taxol is the highest at pH 7.0. Therefore, the pH value of the medium has significant effect on the growth of mycelium and the accumulation of final products. However, the pH in fermentation process is difficult to control. We can only control the initial pH in the fermentation broth. Table 2 showed that when the initial pH was at 5.5, and the seeding culture liquid was cultured at 120 r/min and 28°C, the pH value decreased slowly during the first few days, reaching a pH of 4.08 ± 0.03 . Then the pH value began to increase quickly to 7.78 ± 0.13 at 12 days and to 8.05 ± 0.11 at 16 days after seeding. These results indicate that in the course of fermentation, acid or base were produced. Low or high pH affects the growth of fungi, also leads to an increase in the stickiness of the fermented material and pigment secretion.

Culture temperature

In this study, monofactor experiment was carried out on

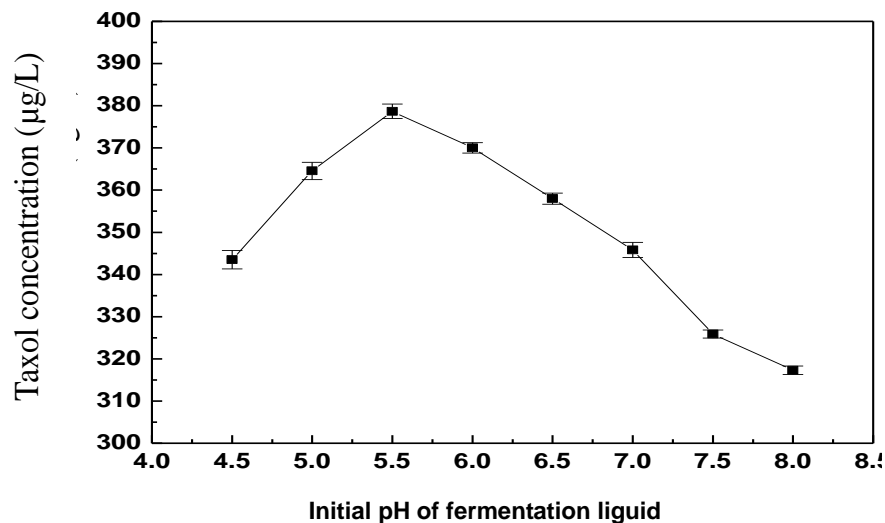


Figure 1. Effect of initial pH of fermentation broth on taxol yield of strain UV₄₀₋₁₉.

Table 2. pH changes in the fermentation process.

Days after inoculation	pH*
0	5.50 ± 0.04
2	4.31 ± 0.05
3	4.23 ± 0.05
4	4.16 ± 0.04
5	4.13 ± 0.05
6	4.08 ± 0.03
7	4.10 ± 0.06
8	4.50 ± 0.05
9	5.07 ± 0.06
10	6.52 ± 0.07
12	7.78 ± 0.13
14	7.85 ± 0.09
16	8.05 ± 0.11

*The experiments were repeated five times and each measured in triplicate. The results were expressed as mean ± SD.

the culture temperature using 22, 25, 28, 30 and 32°C, respectively. The initial pH for the modified S-7 medium was set at 5.5, and the agitation rate was set at 120 r/min for 12 days. Figure 2 showed that when the temperature was 25°C, the concentration of taxol in the fermentation broth reached its maximal at 386.76 ± 2.97 µg/L.

The basic study in metabolic regulation has shown that temperature is closely related to fungus growth. In addition to the reaction rate during fermentation process, temperature can also affect the biosynthetic activities in fungus. The optimal temperature is important to maintain certain enzyme activities. If the temperature is lower than optimum, the reaction rate increases with the increase of temperature, thus improving the metabolic rate and

shortening the production period. When the temperature is higher than the optimum, with the increase of the temperature, the loss of enzyme activities accelerated, causing the shortening of the fermentation cycle and decrease of the final yield of products.

Agitation rate

In this study, the monofactor test on agitation rate was carried out with the rate at 100, 120, 150, 180, 200, 220 and 250 r/min. Figure 3 showed that when the agitation rate was 150 r/min, the yield of taxol was the highest 397.43 ± 3.46 µg/L. The fermentation process for

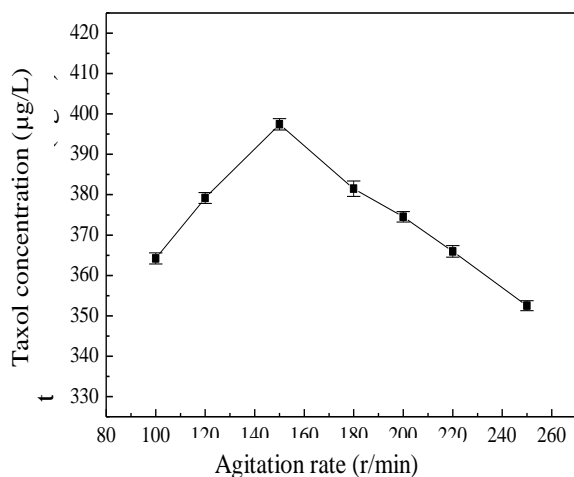
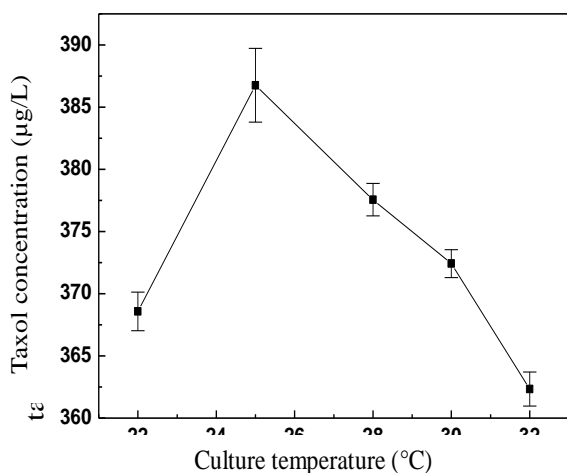


Figure 2. Effect of culture temperature on taxol yield of strain UV₄₀₋₁₉.

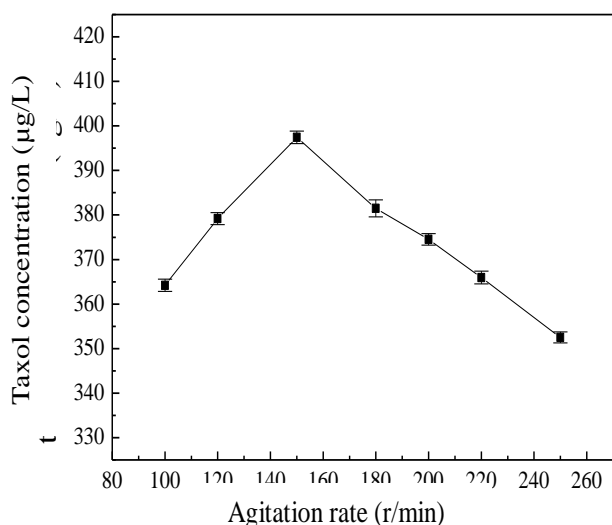


Figure 3. Effect of agitation rate on taxol yield of t strain UV₄₀₋₁₉.

taxol-producing fungi is aerobic. Venting quality can be modulated by changing the agitation rate. Low agitation rate caused small venting effect, which is not helpful for fungus growth. However, too high an agitation rate may cause mycelium to autolyze, thus decrease the biomass production (Chen et al., 2000).

Determination of biomass

The initial pH of the modified S-7 medium was set at 5.5, with agitation rate at 150 r/min and culture temperature at 25°C. From the second day, a certain amount of fermentation broth was drawn daily, and the biomass was measured. Figure 4 showed that the growth of the strain UV₄₀₋₁₉ entered logarithmic growth period directly, with a maximal biomass at 0.097 ± 0.018 g/ml at 6 days after inoculation.

Determination of taxol yield during different periods of culture

After cultured for different periods of time under the conditions described above, the ferment liquid was drawn for taxol extraction. Taxol was then quantified using thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). Table 3 showed that the strain UV₄₀₋₁₉ began to produce taxol at 5 days after inoculation with very a low concentration of 16.31 ± 0.55 µg/L. Then the taxol content increased rapidly, reaching 396.83 ± 2.68 µg/L at 12 days after inoculation (Figure 5A and B). Afterwards, the yield of taxol remained constant or slightly increased. The result from LC-MS analysis showed that the purified product had taxol-specific ion apex m/z 854.5 $[M+H]^+$ (Figure 6).

The typical relationship between the synthesis of metabolic product and the growth of mycelium can be classified as coupling, non-coupling and semi-coupling types. Fett-Neto et al. (1994) suggested that the synthesis of taxol and the growth of *T. cuspidata* cell showed non-coupling relationship, which is also the case for the *T. chinensis* cell culture (Tang et al., 2001). However, Wang et al. (1997) found a semi-coupling relationship between these two events; Mei et al. (1997) suggested that the two events could not be classified as growth-relating or non-growth-relating type. In this study, the synthesis of taxol and the growth of strain UV₄₀₋₁₉ showed semi-coupling relationship, that is, the increase of taxol yield and biomass was not completely synchronous.

Optimization of medium

Production of taxol using biotechnology approaches

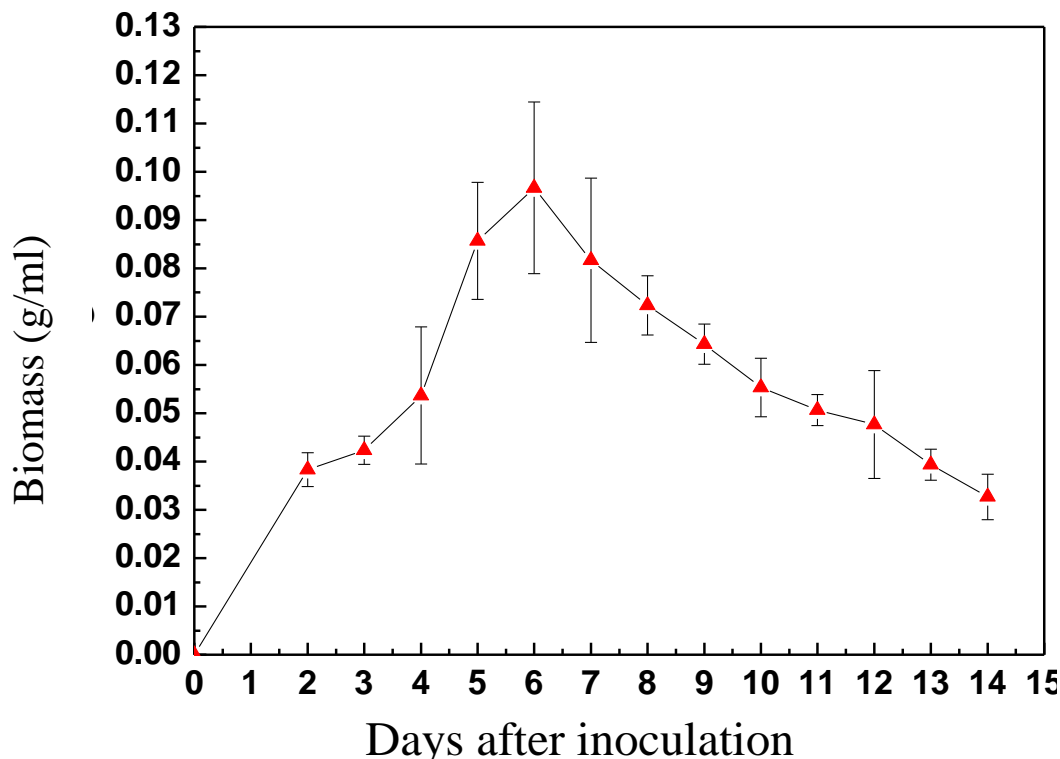


Figure 4. Biomass changes in the fermentation process.

Table 3. Effect of culture time on the yield of taxol.

Days after inoculation	Taxol concentration in the fermentation medium ($\mu\text{g/l}$)*
2	0
3	0
4	0
5	16.31 \pm 0.55
6	52.47 \pm 0.82
7	126.22 \pm 1.05
8	216.84 \pm 0.84
9	338.03 \pm 1.05
10	377.56 \pm 1.82
12	396.83 \pm 2.68
14	397.15 \pm 2.21
16	378.67 \pm 2.04

*The experiments were repeated five times and each measured in triplicate. The results were expressed as Mean \pm SD.

is a major focus in the field of developing taxol resources. The research on using microbe fermentation method has just started. Results obtained with cell cultures has shown that addition of precursors, activators and inhibitors of branching pathways to the medium can modulate taxol

biosynthesis and lead to high yield (Raymond et al., 2007; Fett-Neto et al., 1994; Zhou et al., 2004, 2005; Wang et al., 2007). However, the effect of the compounds on taxol biosynthesis in endofungi has been rarely reported (Xu et al., 2006). Based on the monofactor experiments, this study used L9 (3)4

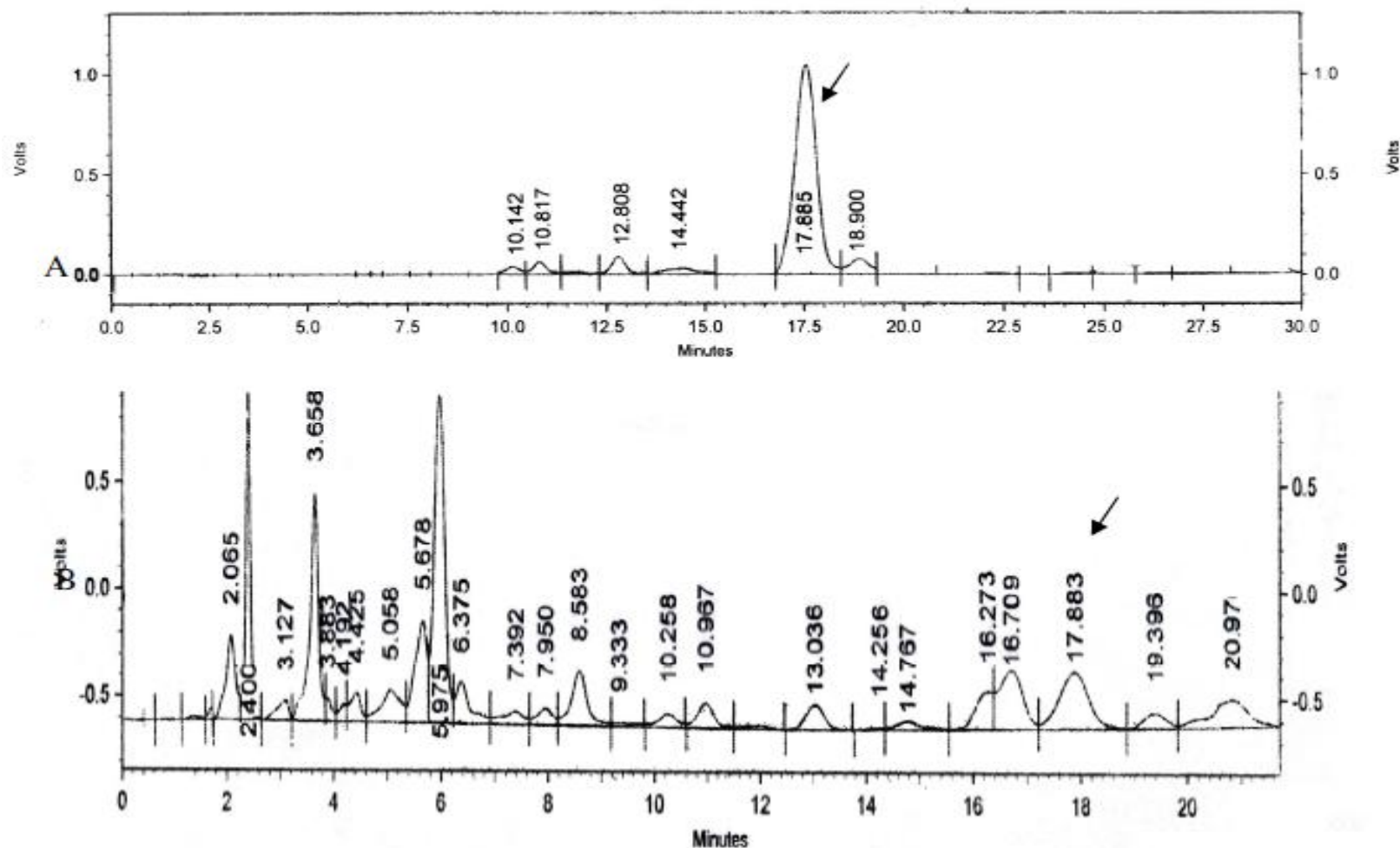


Figure 5. HPLC chromatograms of taxol extracted from strain UV₄₀₋₁₉. Arrows indicate the taxol-specific peaks. A: Taxol sample extracted from strain UV₄₀₋₁₉; B: Taxol molecule standard.

orthogonal design to assess the effect of different concentrations of serine, salicylic acid, silver nitrate and ammonium acetate in the modified S-7 medium on the yield of taxol in UV₄₀₋₁₉. Table 4 showed that the content

of taxol in different fermentation broth was higher than that of the control group ($p < 0.05$), suggesting that effective precursors and inducers in the modified S-7 medium can improve the capability of

UV₄₀₋₁₉ strain to synthesis taxol. The optimal condition for the taxol production is to add serine, salicylic acid, silver nitrate and ammonium acetate with the final concentrations of 5, 80, 8.5 and

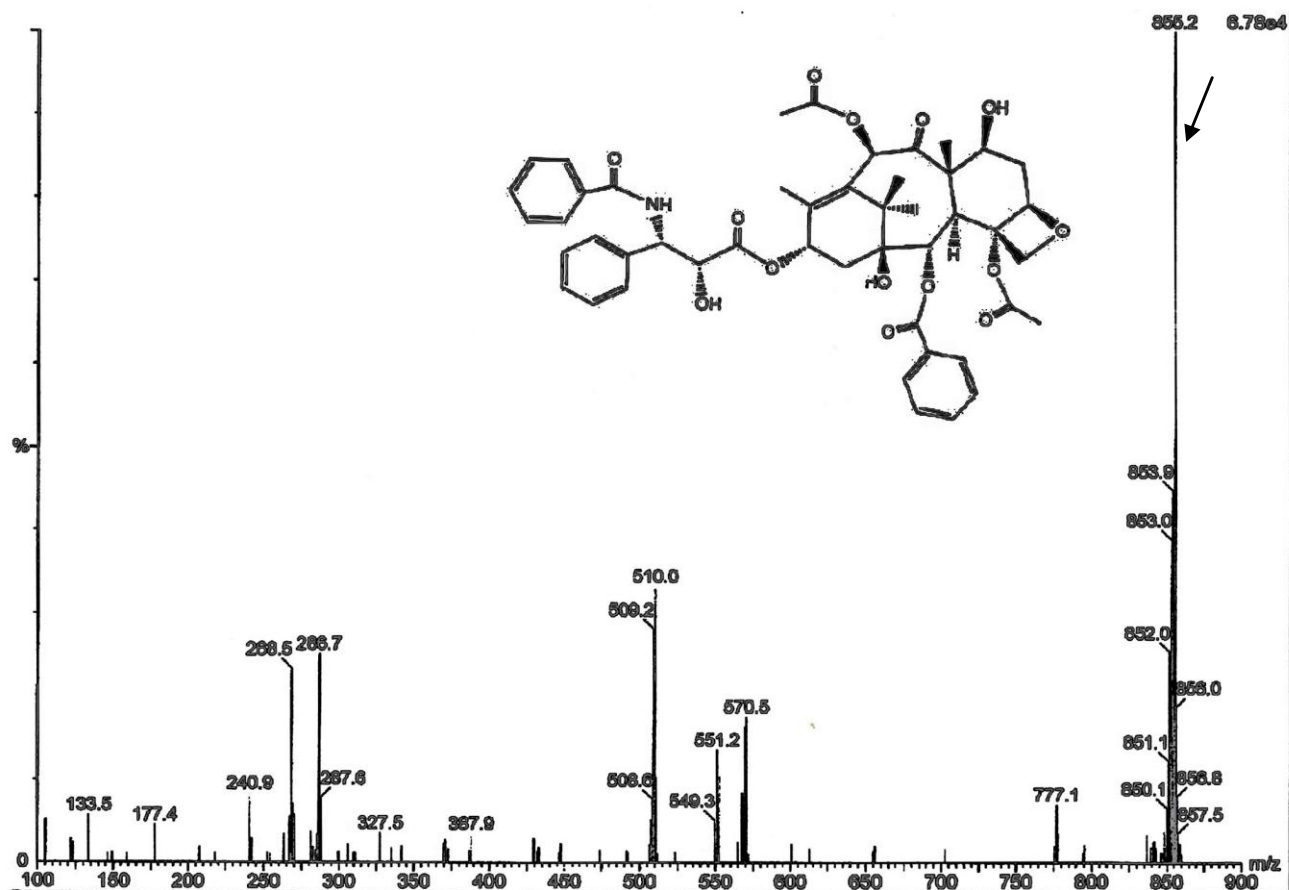


Figure 6. Mass spectrum of taxol extracted from strain UV₄₀₋₁₉. Arrow indicates the molecular ion of taxol at m/z 855 [M+H].

Table 4. Effect of medium composition on taxol production by taxol-producing fungi UV₄₀₋₁₉.

Factors	A	B	C	D	Yield of taxol ($\mu\text{g/L}$)*
	Ammonium acetate (mg/L)	Salicylic acid (mg/L)	Silver nitrate (mg/L)	Serine (mg/L)	
Groups					
1	15	70	0.85	5	406.72 \pm 1.82
2	15	80	1.7	10	414.13 \pm 2.26
3	15	90	8.5	15	407.61 \pm 1.61
4	20	70	1.7	15	423.65 \pm 2.43
5	20	80	8.5	5	456.24 \pm 2.83
6	20	90	0.85	10	437.27 \pm 2.47
7	25	70	8.5	10	424.30 \pm 2.63
8	25	80	0.85	15	418.07 \pm 1.97
9	25	90	1.7	5	411.29 \pm 2.14
Control					397.03 \pm 1.75
X ₁	409.49	418.22	420.69	424.75	
X ₂	439.05	429.48	416.36	425.23	
X ₃	417.89	418.72	429.34	416.44	
R	21.16	10.76	12.98	8.79	

X₁, X₂ and X₃ represent the mean value of factors at 1, 2 and 3 levels. R represents range. *The experiments were repeated five times and each measured in triplicate. The results were expressed as Mean \pm SD.

20 mg/L, respectively. With these additions, the taxol production can be increased from 397.03 ± 1.75 to 456.24 ± 2.83 $\mu\text{g/L}$. According to range value, we found the effect of different factors on the yield of taxol in fermentation broth, which is $A > B > D > C$. Based on statistical principle, the major factor is the most reliable and the optimal level should be used. While for the minor factors, both the optimal and the general levels can be considered. Therefore, the optimal group for the protoplast preparation of *N. sylviforme* is $A_2B_2C_3D_2$, which is the modified S-7 medium supplemented with serine, salicylic acid, silver nitrate and ammonium acetate at the final concentrations of 10, 80, 8.5 and 20 mg/L, respectively.

Conclusions

The optimal condition for taxol production was as follows: the initial pH for the modified S-7 medium was 5.5, with 150 r/min agitation rate at 25°C for 12 days. Other inducers and precursors can be added. Under these conditions, the taxol yield can be increased to 456.24 ± 2.83 $\mu\text{g/L}$, which is 1.15 times as that produced using the S-7 modified medium.

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