Identification and fermentation optimization of a marine-derived

*Streptomyces Griseorubens* with anti-tumor activity

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The purpose is an attempt to investigate a potential anti-tumor actinomycete WBF9 isolated from Chinese marine sediment. MTT assay was firstly used to evaluate anti-tumor activity and ID$_{50}$ value was defined as dilution fold of fermentation broth (FB) that caused 50% inhibition of cell growth. The results reveal its strong anti-tumor activity against Hela, KB and SMMC7721 cells with the ID$_{50}$ values of 750.3, 921.2 and 803.5, respectively. The strain was identified as *Streptomyces griseorubens* according to the 16S rRNA gene sequence analysis, along with the morphological, physiological and biochemical characteristics. The present study also reveals the strain WBF9 required natural seawater for good growth and production of anti-tumor metabolites. This implies some degree of marine adaptation of the strain. The one-factor-at-a-time method was used to investigate the enhanced anti-tumor activity of nutrients. The concentration of the four nutritional components was optimized by the orthogonal matrix method. The effects of the nutritional components for improving anti-tumor activity were found to be in the order of CaCl$_2$> potato extract> yeast extract> glucose and the optimal concentrations were determined as glucose (1% w/v), potato extract (15% w/v), yeast extract (0.6% w/v) and CaCl$_2$ (0.12% w/v). With the optimized medium, ID$_{50}$ value of FB reached the maximum level of 1946.8 in 1-l flask after 7d of fermentation, which was 2.1-fold higher than that with the basal medium. It indicated a significant increase of anti-tumor activity of FB. The ethyl acetate extract was preliminarily isolated and the resulting four fractions showed anti-tumor activity. The initial identification data demonstrated that active fractions contained alkaloid, terpene, peptide, and indican. The strain of *Streptomyces griseorubens* being isolated from the sea was firstly reported here, and its anti-tumor activity was initially investigated in this paper.

**Keywords:** Anti-tumor activity; Identification; ID$_{50}$; Marine actinomycete; Optimization; *Streptomyces griseorubens*

**Introduction**

For several decades, terrestrial actinomycetes have proved to be an important source of biologically active metabolites, including antibiotic, anti-tumor and immunosuppressant agents.$^{1,2,3}$ However, the rate of discovery of new metabolites from terrestrial actinomycetes is diminishing and new sources of bioactive compounds need exploration.$^{1,5}$ Marine actinomycetes have been widely recognized as a potential source of new drug candidates.$^{6,7,8}$ They can produce structurally unique metabolites that are not found in their terrestrial counterparts due to their extreme living conditions within the marine environment.$^{9,10}$ Some novel active compounds from marine actinomycetes are reported at a high frequency.$^{2,11,12}$ and some of them show strong anti-tumor activity, which suggests marine actinomycetes are a promising source of anti-tumor leading drugs.$^9$ Some anti-tumor drugs from marine actinomycetes, such as thiocoraline$^{11}$, salinosporamide$^{14,15}$ and ECO-4601$^{12,16}$, are under clinical trials. Therefore, as a new source of anti-tumor drug candidates, marine actinomycetes have attracted serious attention in the last decade.$^{1,17,18,19}$

The present paper has identified marine actinomycete WBF9 as *S. griseorubens* and reported anti-tumor activity, the requirement of natural seawater and fermentation optimization of the strain based on further developing a testing method of anti-tumor activity of fermentation broth (FB). Also, preliminary identification of active metabolites was carried out.

**Materials and methods**

**Materials and instruments**

Natural seawater (NSW) was collected from the shore of Weihai, China (37°36′N, 121°42′E). Fetal bovine serum (FBS) and RPMI-1640 medium were used to culture the strain. The medium was prepared according to the method of Liang et al.$^{20}$

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obtained from Gibco-BRL (Grand Island, NY). Trypsin, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and LL-DAP (LL-2,6-diaminopimelic acid) was obtained from Sigma-Aldrich Chemical (St. Louis, MO). All other chemicals and reagents were obtained from Shanghai Sangon Biotechnology Co., Ltd (Shanghai, People’s Republic of China).

A microplate reader (Model 680 Microplate Reader; Bio-Rad Laboratories Inc., Sydney, Australia) was used to record the absorbance. A light microscope (BH-2, Olympus, Japan) and a scanning electron microscope (SX-40, Akashi, Japan) were used to observe the fine structure of strain WBF9.

**Microorganism and flask cultures**

The sediment sample was collected by SCUBA diver from the seabed at a depth of about 25 m, 5,000 m away from the shore of Weihai, China (37°36′N, 121°42′E). After collection, the sample was placed in sterile 50-ml plastic bags and kept cool until processed. Strain WBF9 was isolated from the sediment sample using Gause’s Synthetic Argar (soluble starch 20g/l, KNO₃ 1g/l, NaCl 0.5 g/l, KH₂PO₄ 0.5 g/l, MgSO₄•7H₂O 0.5 g/l, FeSO₄•7H₂O 0.01 g/l and agar 17 g/l, 100% NSW) containing potassium dichromate 0.05 g/l.

The stock culture was maintained on Gause’s Synthetic Slants in 25% glycerol solution at -20°C for about 2 months and subcultured every 4 weeks. Slants were incubated at 28°C for 4 days and then stored at 4°C. Strain WBF9 was grown at 28°C on a rotary shaker in GPPY1 medium (1% glucose, 10% potato extract, 0.2% peptone, 0.4% yeast extract, 100% NSW, pH 7.2). After 3d cultivation, the seed culture broth, 5% (v/v), was transferred into GPPY1 medium and fermented at 28°C, 200 rpm for 7d. Culture supernatant was obtained by centrifugation (4,000 g, 15 min) and filtered through a 0.22 µm Millipore Filter. The supernatant samples were tested for anti-tumor activity by MTT assay.

**MTT assay**

Three common human solid tumor cell lines, Hela (human cervical adenocarcinoma), KB (human oral epidermoid cancer) and SMMC7721 (human hepatocarcinoma) were preserved in our lab. These tumor cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were fed with fresh cultured medium 2-3 times per week and subcultured when 80% confluent.

MTT assay was used to investigate in vitro anti-tumor activity of FB against several tumor cell lines according to the previously described method. In brief, tumor cells taken from the exponential phase of cultures were seeded into 96-well plates in 180 µl medium at between 2x10⁴ and 3x10⁴ cells/ml and were incubated at 37°C and 5% CO₂ for 24 h. The supernatant samples were diluted with RPMI 1640 medium. Samples of volume 20 µl, along with 20 µl of fermentation media as the negative control, were then added to the plates in triplicate, giving final dilution fold of 10, 20, 40, 80, 160, 320, 640, 1280, 2560 for the supernatant sample. The cells were incubated for initial period of 44 h, and then with the addition of 20 µl of MTT (5 mg/ml), for another 4 h in the second period. After pouring out the culture medium, 150 µl of DMSO was added to each well to dissolve the formazan product. The absorbance at 490 nm was recorded using a microplate reader. Percentage inhibition rate (IR) was measured by the following equation:

$$IR(\%) = \frac{OD_{control \ well} - OD_{treated \ well}}{OD_{control \ well}} \times 100$$

In the experiment, anti-tumor activity of FB was evaluated with ID₅₀ value. ID₅₀ value represented dilution fold of FB that caused 50% inhibition of cell growth, which was calculated by using the CalcuSyn Version 2.0 according to the IR values of different dilution samples of FB. The higher the ID₅₀ value the stronger is the anti-tumor activity of FB.

**Taxonomic characterization of strain WBF9**

Strain WBF9 was identified according to Bergey's Manual of Systematic Bacteriology and the Taxonomy and Identification of Actinomycetes. Micro-morphological studies were carried out with a light microscope and a scanning electron microscope from the cultures at 28°C for three days on Gause’s synthetic agar medium. Analysis of the whole cells for diaminopimelic acid and carbohydrates were determined according to the methods in the International Streptomyces project (ISP) and Waksman’s media.
**16S rRNA gene sequencing and phylogenetic analysis**

The chromosomal DNA from strain WBF9 was isolated according to the previously described procedure\(^{30}\). The 16S rDNA of the strain was amplified using the 16S rDNA primers, forward primer (5'-CGG AGA GTT TGA TCC TGG CTC AG-3'; positions 5–27, *Escherichia coli* numbering) and reverse primer (5'-AAA GGA GGT GAT CCA GCC GCA-3'; positions 1542–1522, *Escherichia coli* numbering) designed according to previous data\(^{30,31}\) and a conservative part of 16S rDNA of known actinomycete strains (Accession Number: AB026221, AB026220, AB026219, AB022874, etc). The purified PCR product was sequenced using an ABI 3730 sequencer.

The 16S rDNA sequence of strain WBF9 was isolated according to the previously described procedure\(^{30,31}\). The chromosomal DNA from strain WBF9 was the GenBank and the RDP using CLUSTAL X version 1.83\(^{32}\). Phylogenetic trees were constructed using the neighbour-joining, minimum evolution and maximum parsimony methods from MEGA version 3.1\(^{33}\). The genetic distance was calculated with Kimura’s two-parameters model\(^{34}\). The resultant unrooted tree topologies were evaluated by bootstrap analyses based on 5000 replications.

**Effects of NSW on mycelial growth and anti-tumor activity of FB**

To investigate the effects of NSW on mycelial growth and anti-tumor activity of FB against KB cells, NSW in GPPY1 medium was substituted with 100% tap water (TW, GPPY2 medium) or 100% distilled water (DW, GPPY3 medium). Strain WBF9 was inoculated in 250 ml flasks containing 50 ml of three different media at 28°C for 10 h, using 5% (v/v) inocula. After 7d, dry cell weight (DCW) was determined and the supernatant samples were tested for anti-tumor activity.

Optimization of fermentation medium using the one-factor-at-a-time method

In order to study the major nutrients requirement for enhancing anti-tumor activity of FB against KB cells, various carbon sources (glucose, sucrose, fructose, xylose, inositol and potato extract at 2% concentration), nitrogen sources (yeast extract, peptone, meat peptone, KNO\(_3\), NH\(_4\)NO\(_3\) and NH\(_4\)Cl at 0.4% concentration) or mineral sources (KH\(_2\)PO\(_4\), MgSO\(_4\), KCl, CaCl\(_2\), Na\(_2\)HPO\(_4\)•12H\(_2\)O and K\(_2\)HPO\(_4\) at 7 mM concentration) were respectively supplemented to the basal medium (GYPP1 medium) using the one-factor-at-a-time method (Table 1). Fermentation was carried out according to above-mentioned method. After 7d of fermentation, the ID\(_{50}\) values of FB were investigated.

Optimization of concentrations of selected medium components using the orthogonal matrix method

To raise the anti-tumor activity of FB, the orthogonal design L\(_9\)(3\(^4\)) was applied to optimize the concentrations of four selected nutrients (glucose, potato extract, yeast extract and CaCl\(_2\)) in flask experiments\(^{35}\). The level-setting values of the factors used in the orthogonal array design were shown in Table 2. Based on the L\(_9\) orthogonal array design, we carried out 9 experiments in triplicate. After 7d of fermentation, the ID\(_{50}\) values of FB were evaluated.

**Determination of dry cell weight**

FB was withdrawn (20 ml) from the flask at the assigned time. Then, the whole content was centrifuged at 10000xg for 30 minutes. The detached cell pellet was dried in hot air oven at 90°C for 10 h, and DCW was determined.

**Statistical analysis**

The data are shown as mean ± SD. Statistical significance of differences was processed using the Student’s t-test analysis or the variance analysis. Values of P<0.05 were considered significant.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>ID(_{50})</th>
<th>Nitrogen source</th>
<th>ID(_{50})</th>
<th>Mineral source</th>
<th>ID(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1005.6±58.6</td>
<td>Yeast extract</td>
<td>1231.5±85.2</td>
<td>KH(_2)PO(_4)</td>
<td>92.2±9.3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>642.8±35.3</td>
<td>Peptone</td>
<td>453.7±39.5</td>
<td>MgSO(_4)</td>
<td>113.0±13.0</td>
</tr>
<tr>
<td>Fructose</td>
<td>13.5±2.3</td>
<td>Meat peptone</td>
<td>94.8±9.8</td>
<td>KCl</td>
<td>56.4±6.8</td>
</tr>
<tr>
<td>Xylose</td>
<td>11.1±1.8</td>
<td>KNO(_3)</td>
<td>601.6±29.8</td>
<td>CaCl(_2)</td>
<td>1764.3±121.6</td>
</tr>
<tr>
<td>Inositol</td>
<td>31.2±4.2</td>
<td>NH(_4)NO(_3)</td>
<td>53.3±5.3</td>
<td>Na(_2)HPO(_4)•12H(_2)O</td>
<td>94.5±13.5</td>
</tr>
<tr>
<td>Potato extract</td>
<td>1325.3±79.8</td>
<td>NH(_4)Cl</td>
<td>56.3±4.7</td>
<td>K(_2)HPO(_4)</td>
<td>25.9±3.5</td>
</tr>
</tbody>
</table>

The values of ID\(_{50}\) were mean±SD from three independent experiments.
Results

Anti-tumor activity of FB from strain WBF9

With the GPPY1 medium, anti-tumor activity of FB from strain WBF9 were investigated against three tumor cell lines. FB exhibited strong activity against Hela cells, KB cells and SMMC7721 cells with the ID$_{50}$ values of 750.3, 921.2 and 803.5, respectively, indicating strain WBF9 was a promising source of anti-tumor compounds. In the following anti-tumor assay, KB tumor cell line was used as bioassay-directing tumor cell line owning to its sensitivity to active FB.

Identification of strain WBF9

The fine structure of strain WBF9 was mainly studied using light microscope (× 400; Fig. 1a) and scanning electron microscope (× 7000; Fig. 1b). The spore chains were regularly spiral, with four to eight turns per chain and more than 10 spores per chain. All spores were spiny on the surface and the shape varied from spherical to ovoid. The spore diameter varied from 0.5 to 1.0 µm and length varied from 0.6 to 1.4 µm. These characteristics were similar to those of *Streptomyces griseorubens* (*S. griseorubens*).25

Thin-layer chromatography showed that only LL-DAP was found in the whole cell hydrolysate, but no characteristic sugars were present. Thus, strain WBF9 had the same composition of symbolic cellular components as the genus *Streptomyces*.24

The cultural characteristics of strain WBF9 on typical agar media for actinomycetes (namely, yeast malt agar, oatmeal agar, starch inorganic salt agar, peptone yeast iron agar, tyrosine agar, glucose sparagines agar, gause’s synthetic agar, potato piece) showed that the strain possessed similar characteristics to *S. griseorubens*. It grew well on the agar media and the spore mass was found to be gray in color without pigment secreted into media.

The physiological and biochemical properties of strain WBF9 also were very similar to those of *S. griseorubens*. Liquefaction of gelatin, coagulation and peptonization of milk, hydrolysis of starch and decomposition of cellulose were all positive, but formation of melanin, H$_2$S and nitrite was negative. Both strains consumed D-glucose, D-xylose, L-rhamnose, D-fructose and mannitol, but couldn’t utilize sucrose and raffinose.

The nearly complete 16S rDNA sequence of strain WBF9 was generated (1450 nt, Genbank accession no. EF010983). Phylogenetic analysis of 16S rDNA sequences of the strain and related taxa showed almost-complete 16S rDNA sequence of the strain had 100% similarity to that of *S. griseorubens* (Genbank accession no. AB184139) (Fig. 2). As a result, strain WBF9 was identified as *S. griseorubens*.

Effects of NSW on mycelial growth and anti-tumor activity of fermentation broth

To determine whether NSW could affect mycelial growth and anti-tumor activity, strain WBF9 was fermented in GPPY1, GPPY2 and GPPY3 media. After 7d, DCW and ID$_{50}$ values of FB were investigated. DCW value in GPPY1 was 30.8±6.3 g/L, which was significantly higher compared with the values in GPPY2 and GPPY3 (15.1±4.3 g/L and 14.8±5.2 g/L) (p<0.01). At the same time, FB in GPPY1 medium exhibited strong anti-tumor activity against KB cells (ID$_{50}$=915.3±69.4), while FB in GPPY2 and GPPY3 had no anti-tumor activity. The results indicated that NSW rather than TW and DW...
could stimulate fast growth of strain WBF9 and production of anti-tumor metabolites.

Optimization by the one-factor-at-a-time method

With the one-factor-at-a-time method, the effects of carbon sources, nitrogen sources and mineral sources on anti-tumor activity against KB cells were tested. Among all carbon sources tested, both potato extract ($\text{ID}_{50}=1325.3\pm79.8$) and glucose ($\text{ID}_{50}=1005.6\pm58.6$) had a beneficial effect on anti-tumor activity of FB, while yeast extract ($\text{ID}_{50}=1231.5\pm85.2$) and CaCl$_2$ ($\text{ID}_{50}=1764.3\pm121.6$) were the most suitable nitrogen source and mineral source, respectively (Table 1). As a result, the concentrations of the four nutrients were optimized by the $L_9(3^4)$ orthogonal matrix in the following experiment.

Experimental results of $L_9(3^4)$ orthogonal matrix method

The $L_9(3^4)$ orthogonal matrix method was often used to optimize the nutritional factors in the fermentation experiments$^{35}$. In our study, four factors (potato extract, glucose, yeast extract and CaCl$_2$) and three levels of every factor were showed in Table 2. The fermentation results of $\text{ID}_{50}$ values against KB cells by the orthogonal matrix method were given in Table 2. The intuitive analysis (Table 2) and the variance analysis results (Table 3) indicated that $\text{ID}_{50}$...
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The ID$_{50}$ of FB could be enhanced using a combination of those factors at different levels. Among the four nutrients, CaCl$_2$ was the most significant effect factor on ID$_{50}$ of FB. Using the orthogonal design L$_9(3^4)$ approach, the relationships between the factors could be calculated. The factors could be ranked in importance as follows: CaCl$_2$>potato extract>yeast extract>glucose. Table 2 and Table 3 presented the optimal combinations and levels required to achieve the highest ID$_{50}$, namely (w/v): glucose 1%, potato extract 15%, yeast extract 0.6%, CaCl$_2$ 0.12%. Therefore, the combined medium of four nutrients was used as the optimized medium for fermentation.

**Fermentation results in shake-flask experiments**

With the orthogonal matrix method, the optimized medium combination was obtained. To determinate if the ID$_{50}$ of FB in the optimized medium was higher than that in the basal medium, time course fermentation was carried out in a 1-L flask containing 400 ml basal medium or optimized medium (Fig. 3). Fermentation run was implied according to the above methods.

Fermentation results were given in Fig. 3. In a basal medium (Fig. 3a), the ID$_{50}$ value against KB cells reached a maximum level of 919.2, and maximum DCW was 30.2 g/L at 7d. Under the optimized medium (Fig. 3b), the maximum ID$_{50}$ value against KB cells was 1946.8 at 7d, which was 2.1-fold higher than that with the basal medium, and DCW was 37.8 g/L, indicating anti-tumor activity of FB was significantly raised.

**Discussion**

In the present study, FB of marine actinomycete WBF9 was found to possess strong anti-tumor activity against three tumor cell lines. The strain was further identified as *S. griseorubens* according to the traditional classification and the 16S rRNA gene sequence analysis. Initially, *S. griseorubens* was reported to be isolated from the land in the 1950’s $^{36}$. However, no references pointed out that *S. griseorubens* could produce anti-tumor metabolites. In this study, we firstly reported the *S. griseorubens* being isolated from the sea and demonstrated its anti-tumor activity.

Since *S. griseorubens* WBF9 lived in extreme marine environment different from the land, the ecological differentiation could lead *S. griseorubens* to produce active metabolites. Our finding may be explained by the previous reports that each metabolite may have a distinct ecological function $^{11,37}$. The results in this study will attract more attention on the investigation of *S. griseorubens* WBF9 isolated from the sea as a potential source of anti-tumor drugs.

When investigating the effects of NSW on mycelial growth and anti-tumor activity, we observed that

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**Table 2—Factors and levels setting of the experiment in the L$_9$(3$^4$) matrix and the intuitive analysis of experiments**

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Glucose A (%)</th>
<th>Potato extract B (%)</th>
<th>Yeast extract C (%)</th>
<th>CaCl$_2$ D (%)</th>
<th>The ID$_{50}$ value of FB against KB cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>5</td>
<td>0.2</td>
<td>0.04</td>
<td>773.6 ± 45.5</td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>10</td>
<td>0.4</td>
<td>0.08</td>
<td>1623.7 ± 110.5</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>15</td>
<td>0.6</td>
<td>0.12</td>
<td>1886.3 ± 84.1</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>5</td>
<td>0.4</td>
<td>0.12</td>
<td>1631.4 ± 67.1</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>10</td>
<td>0.6</td>
<td>0.04</td>
<td>1411.7 ± 49.7</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>15</td>
<td>0.2</td>
<td>0.08</td>
<td>1630.4 ± 58.7</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>5</td>
<td>0.6</td>
<td>0.08</td>
<td>1264.2 ± 68.0</td>
</tr>
<tr>
<td>8</td>
<td>1.5</td>
<td>10</td>
<td>0.2</td>
<td>0.12</td>
<td>1651.9 ± 65.5</td>
</tr>
<tr>
<td>9</td>
<td>1.5</td>
<td>15</td>
<td>0.4</td>
<td>0.04</td>
<td>1283.7 ± 16.3</td>
</tr>
</tbody>
</table>

K1 = 1427.7 1223.1 1351.9 1156.3
K2 = 1557.8 1562.3 1512.8 1506.0
K3 = 1399.9 1600.1 1520.7 1723.2
R = 157.9 377.0 168.8 566.9

Optimal level 2 3 3 3

The assignments of column A, B, C, and D were performed by orthogonal array consisted of nine experiments corresponding to the nine rows and four columns. The values of ID$_{50}$ were mean±SD from three independent experiments. $^a$Average of the sum at the same level. $^b$R=max(average of k$_i$)-min(average of k$_i$).

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**Table 3—The variance analysis of L$_9$(3$^4$) orthogonal test on optimization of different factors**

<table>
<thead>
<tr>
<th>Sources of variance</th>
<th>Sum of Squares</th>
<th>Degree of freedom</th>
<th>Mean Square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>127880.1</td>
<td>2</td>
<td>63940.0</td>
<td>14.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>B</td>
<td>775974.1</td>
<td>2</td>
<td>387987.0</td>
<td>85.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C</td>
<td>163290.0</td>
<td>2</td>
<td>81645.0</td>
<td>17.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>D</td>
<td>1472402.4</td>
<td>2</td>
<td>736201.2</td>
<td>161.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Error</td>
<td>81930.4</td>
<td>18</td>
<td>4551.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>60319892.6</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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strain WBF9 grew well and produced anti-tumor metabolites in the medium containing NSW, rather than TW and DW. NSW is a complex solution containing a wide variety of organic and inorganic chemicals. It could provide some beneficial nutrients for mycelial growth and production of active metabolites. But when we tried to investigate which nutrients in NSW resulting in the fast growth and anti-tumor activity of strain WBF9, we had some difficulty due to the complexity of NSW components. It is possible that organic matter, inorganic matter or combination of them induced the effects. To some extent, the strain had adapted to life in the sea and could be considered facultative marine actinomycete.

In the evaluation of anti-tumor activity of FB by MTT assay, we further developed a testing method by defining a parameter $\text{ID}_{50}$ to assess the anti-tumor activity. Briefly, the IR values of two-fold serial dilutions of every supernatant were firstly determined. Then, $\text{ID}_{50}$ value of the supernatant was calculated with CalcuSyn software according to the serial IR values. Experiments showed that $\text{ID}_{50}$ value could exactly evaluate anti-tumor activity of FB. For example, in optimization of fermentation medium with the one-factor-at-a-time method, IR values of the original FB against KB cells were 93%±5.6% and 95%±8.3% (data not shown) when glucose and potato extract was respectively added to the basal medium. There was no significant difference between the two IR values ($p>0.05$). However, $\text{ID}_{50}$ values of the both FB (1005.6±58.6, 1325.3±79.8) (Table 1) showed significant difference ($p<0.05$). Similarly, the phenomenon in orthogonal matrix experiment was observed. All the data indicated that our developed parameter $\text{ID}_{50}$ was reliable and applicable for evaluating anti-tumor activity of FB, especially in fermentation optimization.

Generally, nutrient components of medium need to be optimized to obtain more objective products. Using the one-factor-at-a-time method and the $L_9(3^4)$ orthogonal matrix method, we obtained the optimized medium. When carrying out time course fermentation, we validated that anti-tumor activity of FB was much higher under the optimized medium than under the basal medium (Fig. 3). The results indicated that the orthogonal matrix method used in this work was an efficient method for optimization of fermentation medium.

Our study on the $S.\text{ griseorubens}$ WBF9 isolated from the sea demonstrated its higher anti-tumor properties and a great potential source of anti-tumor compounds. The metabolites from strain WBF9 will be intensively investigated in our further study.

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**References**


