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# 具抗肿瘤活性放线菌菌株 YIM 90022 的分离和系统发育分析

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**摘 要**:从青海盐碱土壤样品中分离到一株兼性嗜碱放线菌 YIM 90022,该菌株的发酵产物具有很强的体外抗胃癌、肺癌、乳腺癌、皮肤癌、肾癌和子宫癌肿瘤细胞株活性。基于 16S rRNA 基因序列的系统发育分析表明,菌株 YIM 90022 属于拟诺卡氏属(*Nocardiopsis*)的成员,与该属的 4 个有效发表种 *N. exhalans* DSM 44407$^T$,*N. prasina* DSM 43845$^T$,*N. metallicus* DSM 44598$^T$ 和 *N. listeri* DSM 40297$^T$ 系统发育关系最密切,与其分别以 98.8%,98.5%,98.4% 和 97.8% 的 16S rRNA 基因核苷酸序列相似性聚为一簇。但菌株 YIM 90022 不与这 4 个有效种中任何一个单独相聚,形成了一个独立亚分枝。结合形态特征、生理生化特性、细胞化学分类特征,以及 rep-PCR 基因指纹分析等方面的研究结果,菌株 YIM 90022 可能为拟诺卡氏菌属的一个潜在新种。菌株 YIM 90022 在大多数培养基上生长良好,气生菌丝和基内菌丝丰富,在酵母膏麦芽膏琼脂、燕麦片琼脂等培养基中产生可溶性色素。生长 pH 范围 6.0 ~ 12.0,最适 pH 8.5;能在含 0 ~ 15 % NaCl (W/V) 的培养基上生长。

**关键词**:拟诺卡氏菌属(*Nocardiopsis*);抗肿瘤活性;16S rRNA 基因;Rep-PCR 基因指纹分析;系统发育分析

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拟诺卡氏菌属(*Nocardiopsis*)菌株近年来被广泛发现于中高盐环境中[1~4]。我室在对新疆、青海等地的盐碱环境微生物资源调查研究中,也分离到很多拟诺卡氏菌属菌株,其中有不少新物种[6~8],并且发现有些菌株的发酵产物具有多种生物活性。本文报道一株具有很强抗肿瘤活性的拟诺卡氏菌属菌株 YIM 90022 的分离和系统发育分析结果。

## 1 材料和方法

### 1.1 材料

**1.1.1 菌株**:拟诺卡氏菌属典型菌株 *N. exhalans* DSM 44407$^T$,*N. listeri* DSM 40297$^T$,*N. metallicus* DSM 44598$^T$,*N. prasina* DSM 43845$^T$,*N. tropica* DSM 44381$^T$ 和 *N. umidischolae* DSM 44362$^T$ 由本室保藏。实验菌株 YIM 90022 分离自青海盐碱土壤。

**1.1.2 培养基**:HV 琼脂培养基[9]、ISP 系列琼脂培养基[10] 按相关文献配制,pH 7.5;marine agar 2216 (MA)和 marine broth(MB)培养基购自 Difco 公司;发酵培养基组成:葡萄糖 20g,甘露醇 20g,大豆粉 20g,蛋白胨 2g,NaCl 20g,$CaCO_3$ 3g,$(NH_4)_2SO_4$ 5g,$MgSO_4 \cdot 7H_2O$ 0.5g,$KH_2PO_4$ 0.2g,水 1000mL,pH 7.5。

**1.1.3 主要试剂和仪器**:PCR 引物、PCR 常规操作用试剂和酶均购自 TaKaRa 公司;用 TaKaRa MiniBEST Bacterial Genomic DNA Extraction Kit 试剂盒提取和纯化 rep-PCR 所用 DNA;1kb DNA Ladder 购自上海生物工程公司;PCR 仪为 PE-9600 型;凝胶成像系统及成像软件 GeneSnap 3.0 购自 SynGene 公司;UV-1601 分光光度仪购自 Shimadzu 公司。

### 1.2 菌种分离和培养

从青海采集盐碱土壤样品,风干,120℃ 处理 1h,采用稀释涂布平板法,用含 10% NaCl(W/V)的 HV 琼脂培养基进行菌种分离。经纯化后,用含 2% NaCl(W/V)的 ISP2 琼脂培养基和 MA 培养基培养。菌种制成冻干牛奶管,或悬浮于添加 20% 甘油的 MB 培养基中,保藏于 4℃。

### 1.3 菌株抗肿瘤活性测定

**1.3.1 样品制备**:刮取一支成熟的斜面孢子,接种于装有 80mL 发酵培养基的 500mL 三角瓶中,

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200r/min,28℃培养 96h。离心后上清加入等体积萃取夜(乙酸乙酯:甲醇:丙酮 = 2:2:1),重复萃取 2 次,有机相合并蒸馏浓缩。浓缩液冻干,邮寄给协作单位进行生物活性筛选。

**1.3.2 生物活性测定**:由德国 Oncotest 公司协作完成。主要筛选模型有胃癌(Gastric cancer)和肺癌(Lung cancer)高阻抗肿瘤细胞株、乳腺癌(Mammary cancer)和皮肤癌(Melanoma cancer)中阻抗肿瘤细胞株、肾癌(Renal cancer)和子宫癌(Uterus cancer)敏感肿瘤细胞株。

### 1.4 表型特征和化学分类特征

形态观察、培养特征、生理生化特性和细胞化学分析按 Li 等[7] 所用的方法进行。所有培养基补充 2% NaCl (W/V)。按 Hopwood 等的方法提取总 DNA[11],用热变性法测定 G + C 含量[11]。

### 1.5 16S RNA 基因序列测定和系统发育分析

总 DNA 的提取、16S rRNA 基因的 PCR 扩增和测序按 Cui 等[13] 使用的方法进行。根据测序结果,用 Blast 搜索软件从 GenBank、EMBL 等数据库中调出相似性较高的相关放线菌菌株的 16S rRNA 基因序列,用 CLUSTAL X[14] 进行多序列比对,并采用 Saitou 和 Nei[15] 的邻接法 Neighbor-Joining)进行系统进化树的构建和同源性比较。

### 1.6 rep-PCR 基因指纹分析

按试剂盒说明提取和纯化 DNA。引物为 BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′),按 Angela 等[16] 的方法进行 PCR 扩增。用 1kb DNA Ladder 作 marker,取 9 ~ 11μL PCR 产物用 2 %(W/V)琼脂糖凝胶 120V 电泳 150 ~ 240min,用 EB(ethidium bromide)染色。用 GeneTools 2.11(SynGene)进行图象分析。按 Scortichini 等[17] 的方法把带型转换成二进制距阵,用 NTSYS 2.02(Exeter Software, New York, USA)软件分析 rep-PCR 基因指纹图谱,用非加权平均连锁法(Unweighted pair group method using averages algorithm, UPGMA)进行聚类分析,构建树状图。用 Dice 参数计算相似性[18]。

## 2 结果

### 2.1 菌株抗肿瘤活性

筛选结果表明,菌株 YIM 90022 的发酵产物具有很强的体外抗胃癌、肺癌、乳腺癌、皮肤癌、肾癌和子宫癌肿瘤细胞活性。

### 2.2 形态和培养特征

菌株 YIM 90022 具有典型的拟诺卡氏属的特征,在大多数培养基上生长良好,气生菌丝和基内菌丝均很丰富,白色至深棕色。气生菌丝中等分枝,成长短不一的孢子链,孢子杆状,表面光滑;基内菌丝多分枝,常断裂成不规则杆状。在酵母膏麦芽膏琼脂(ISP 2)、马铃薯浸汁琼脂和 ISP 6 中产生棕色至深棕色可溶性色素。菌株 YIM 90022 的培养特征见表 1,显微形态特征见图 1。

**表 1 菌株 YIM 90022 的培养特征**

Table 1 Cultural characteristics of strain YIM 90022

| Medium* | Growth | Aerial mycelium | Substance mycelium | Soluble pigment |
|---|---|---|---|---|
| Yeast extract/malt extract agar (ISP 2) | Good | Light gray | Deep brown | Deep brown |
| Oatmeal agar (ISP 3) | Abundant | Light gray | Light gray | Deep brown |
| Inorganic salt/starch agar(ISP 4) | Abundant | Light gray | Light gray | – |
| Glycerol/asparagines agar(ISP5) | Poor | Light gray | Pale gray | Light gray |
| Peptone-yeast ext-Fe agar(ISP6) | Good | White | Light brown | Light brown |
| Czapek's agar | Good | Yellowish white | Yellowish white | Black brown |
| Potato extract agar | Moderate | Yellowish white | Light yellow | – |

* All media were adjusted to pH 8.5, supplemented with 2% NaCl (W/V); –, No soluble pigment was produced; ISP, International Streptomyces Project[10].

### 2.3 系统发育分析

测得菌株 YIM 90022 的 16S rRNA 基因核苷酸序列(GenBank accession DQ387958)全长 1500 bp。将其与从 GenBank 等数据库调集的拟诺卡氏属以及相关属菌株的 16S rRNA 基因序列进行比较,采用 Clustsl X[14] 进行多序列匹配比对,通过 Mega 2.1 软件进行系统进化树的构建(图 2)。它是根据 Neighbor-Joining 法[15] 和 Kimur[19] 双参数矫正模型建立起来的,通过 1000 次取样计算其 Bootstrap 值(Bootstrap 值标注在图上)。结果表明,菌株 YIM 90022 与拟诺卡氏属的 *N. exhalans* DSM 44407$^T$, *N. prasina* DSM 43845$^T$, *N. metallicus* DSM 44598$^T$ 以及 *N. listeri* DSM 40297$^T$ 系统发育关系最密切,与其分别以 98.8%, 98.5%, 98.4% 和 97.8% 的16S rRNA 基因核苷酸序列相似性聚为一簇。但是,菌株 YIM 90022 不与这 4 个有效种中任何一个单独相聚,形成

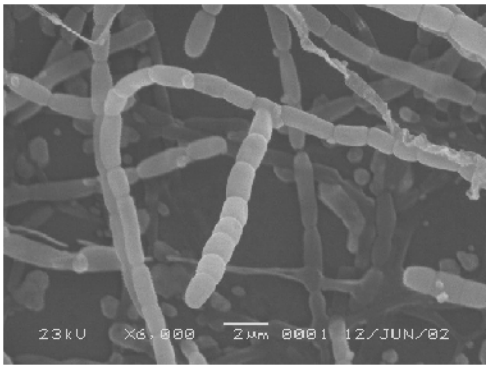


**图 1 菌株 YIM 90022 在含 2 % NaCl(W/V)酵母膏-麦芽膏琼脂培养基培养 21d 的扫描电镜照片**

Fig. 1 Scanning electron micrograph of strain YIM 90022 on ISP 2 supplemented with 2 % NaCl (W/V) for 21 days.

了一个独立亚分枝,提示该菌株可能是一个潜在新种。在拟诺卡氏菌属中,有些有效发表种之间的 16S rRNA 基因序列相似性甚至高于 99 %,例如 *N. tropica* DSM 44381$^T$ 和 *N. umidischolae* DSM 44362$^T$ 之间(99.2 %),*N. metallicus* DSM 44598$^T$ 和 *N. exhalans* DSM 44407$^T$ 之间(99.5 %),*N. dassonvillei* subsp. *Dassonvillei* DSM 43111$^T$ 和 *N. synnemataformans* DSM 44143$^T$ (99.4%),或 *N. metallicus* DSM 44598$^T$ 和 *N. prasina* DSM 43845$^T$ (99.4%)之间。

### 2.4 rep-PCR 基因指纹分析

采用 BOXA1R 引物对菌株 YIM 90022 和与其系统发育关系比较密切的拟诺卡氏菌属典型菌株 *N. exhalans* DSM 44407$^T$, *N. listeri* DSM 40297$^T$, *N. metallicus* DSM 44598$^T$, *N. prasina* DSM 43845$^T$, *N. tropica* DSM 44381$^T$ 和 *N. umidischolae* DSM 44362$^T$ 的基因组 DNA 进行了 rep-PCR 扩增和电泳分离,图谱见图 3-A。可以看出,各泳道在 250 ~ 3500 bp 之间有多条清晰电泳带,其中几条共同的特异条带一定程度上揭示了这些菌株之间较密切的系统发育关系。菌株 YIM 90022 共有 12 条带,其中 6 条为特有带,说明其带型与其它菌株的带型有较大差异。对各菌株 rep-PCR 基因指纹图谱进行聚类分析,构建树状图如图 3-B。菌株 YIM 90022 与其它 6 个与其系统发育关系比较密切的拟诺卡氏菌属典型株之间较大基因型差异,可视为不同基因种[20]。

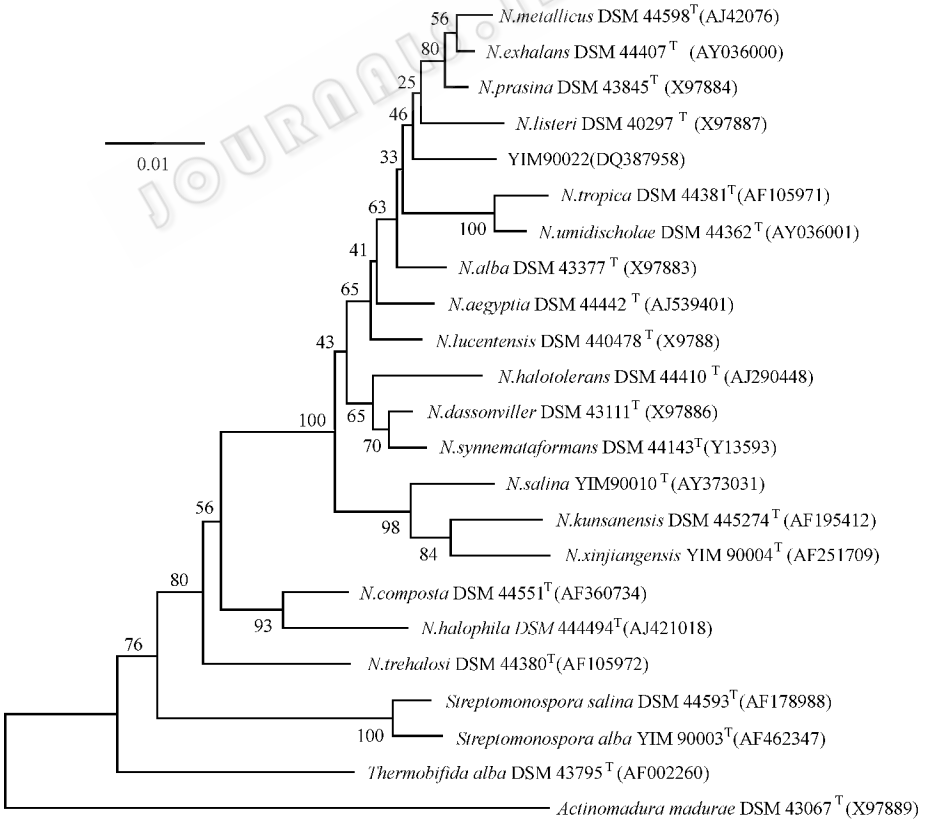


**图 2 菌株 YIM 90022 及其从 GenBank 等数据库中调集的相关属种构建的以 16S rRNA 基因序列为基础的系统树状关系图**

Fig. 2 Neighbor-Joining tree constructed showing the phylogenetic relationships among strain YIM 90022 and other related strains downloaded from GenBank etc. Numerals on branches are the supporting percentage by 1000 replicates. Bar, 1 nucleotide substitution per 100 nucleotides of 16S rRNA gene sequence.

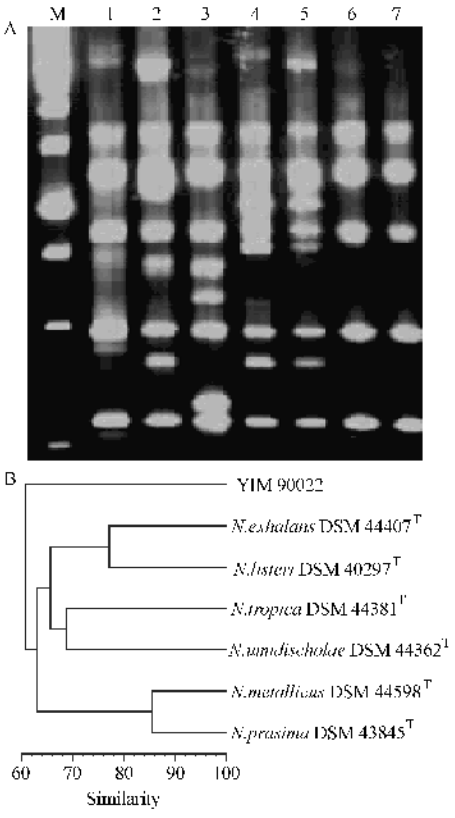


**图 3 菌株 YIM 90022 和与其系统发育关系密切的拟诺卡氏菌属典型菌株的 rep-PCR 基因指纹图谱(A)和基于指纹图谱构建的相互关系树状图(B)**

Fig.3 Rep-PCR fingerprinting patterns from genomic DNA of stain YIM 90022 and 6 related type strains of the genus *Nocardiopsis* (A), and the dendrogram of relationships among strain YIM 90022 and its relatives, derived from rep-PCR DNA fingerprinting. A similarity matrix was produced from analysis of rep-PCR data obtained using BOXA1R primer. Cluster analysis was performed by UPGMA on matrix calculated with the Dice's coefficients (B). M, 1kb DNA ladder; 1. YIM 90022; 2. N. exhalans DSM 44407$^T$; 3. *N. listeri* DSM 40297$^T$; 4. *N. metallicus* DSM 44598$^T$; 5. *N. prasima* DSM 43845$^T$; 6. *N. tropica* DSM 44381$^T$; 7. *N. umidischolae* DSM 44362$^T$.

### 2.5 生理生化特性

菌株 YIM 90022 能在较高碱性环境中生长,酸碱耐受范围 pH6.0~12.0,最适生长 pH8.5;能在含 0~15% NaCl(W/V)的培养基上生长;在 20~45℃生长良好,不能在 4℃或 55℃生长。能分解 Tween 20 和淀粉,不能分解 Tween 80、酪素、纤维素和明胶。硝酸盐还原阳性,吲哚产生、黑色素产生和 $H_2S$ 产生阴性。能利用 D-fructose, glycerol, D-mannose, Raffinose, D-ribose, sorbitol, sucrose, D-trehalose 和 D-xylose 能利用 L-asparagine, L-glutamic acid, L-glycine, L-histidine 和 L-serine 做氮源;不能利用 D-arabinose, cellobiose, dulcitol, D-galactose, D-glucose, inositol, lactose, mannitol, D-maltose, L-Rhamnose, D-xylitol 等碳源和 L- alanine, L-arginine, L-cystine, L-methimine, L-phenylalanine, L-threonine 和 L-valine 等氮源。菌株 YIM 90022 和与其在系统发育上最近的 4 个种典型菌株之间的生理生化特征等表型差异显著(表 2)。

### 2.6 细胞化学特征

菌株 YIM 90022 的细胞壁含 *meso*-DAP (*meso*-diaminopimelic acid)(胞壁Ⅲ型)。全细胞水解物不含特征性糖。主要有磷脂酰胆碱(phosphatidylcholine, PC)磷脂酰甘油(phosphatidylglycerol, PG),二磷脂酰甘油(diphosphatidylglycerol, DPG)和磷脂酰甲基乙醇胺(phosphatidylmethylethanolamine, PME)等磷酸类脂。优势醌(predominant menaquinone)为 MK-10($H_4$, $H_6$)。基因组 DNA 的 G+C mol% 含量为 71.5。

## 3 讨论

根据以上形态学、培养特征、生理生化和系统发育分析的研究结果,菌株 YIM 90022 具有典型的拟诺卡氏菌属的特征,应归属于此属。菌株 YIM 90022 与拟诺卡氏菌属的 4 个有效发表种 *N. exhalans* DSM 44407$^T$, *N. prasina* DSM 43845$^T$, *N. metallicus* DSM 44598$^T$ 和 *N. listeri* DSM 40297$^T$ 系统发育关系最密切,与其以较高的 16S rRNA 基因核苷酸序列相似性(98.1%~98.8%)在以 16S rRNA 基因序列分析为基础的系统发育树状图中聚为一簇。但是,该菌株不与这 4 个种中任何一个单独相聚,而是独立形成了一个亚分枝。rep-PCR 基因指纹分析进一步揭示了菌株 YIM 90022 与其它菌株间存在较大的基因型差异。而且,菌株 YIM 90022 在形态、培养特征、耐盐碱特性、物质同化分解方面,以及磷酸类脂、优势琨组成等方面又与这 4 个种有明显差异(表 2)。所以,根据这些结果,把菌株 YIM 90022 归入拟诺卡氏属中任何种都不太恰当,可以初步确定其为拟诺卡氏菌属的一个种一级新分类单元。当然,要最终确定其分类地位,需补充该菌株和与其系统发育关系密切的典型菌株的 DNA-DNA 杂交等实验。该菌株的发酵产物具有很强的体外抗多种肿瘤细胞株活性,提示从极端环境中筛选生物活性物质产生菌是一条有效途径。

**表 2 菌株 YIM 90022 与相近的拟诺卡氏属典型种特征的比较**

Table 2 Differential characteristics between strain YIM 90022 and closely related *Nocardiopsis* species

| Characteristics | YIM 90022 | *N. exhalans* DSM 44407$^T$ | *N. prasina* DSM 43845$^T$ | *N. metallicus* DSM 44598$^T$ | *N. listeri* DSM 40297$^T$ |
|---|---|---|---|---|---|
| Catalase activity | + | + | + | ND | + |
| Growth at： | | | | | |
| 10 ℃ | + | + | − | W | + |
| 45 ℃ | + | − | − | − | − |
| pH 12 | + | ND | − | − | ND |
| 10 % NaCl | + | + | + | + | − |
| Utilization of ： | | | | | |
| L-Alanine | + | + | + | − | − |
| L-Arabinose | − | + | + | ND | + |
| D-Fructose | + | + | − | ND | + |
| D-Galactose | W | − | − | + | − |
| Gelatin | − | ND | + | + | + |
| D-Glucose | + | + | − | + | + |
| L-Histdine | + | − | + | ND | + |
| D-Maltose | − | + | + | + | + |
| Mannitol | W | ND | − | − | − |
| D-mannose | + | + | − | + | + |
| L-Phenylalanine | − | + | + | ND | + |
| L-proline | + | + | − | + | + |
| Raffinose | + | ND | − | − | − |
| L-Rhamnose | − | + | − | + | + |
| L-Serine | + | + | − | − | − |
| Sucrose | + | ND | − | + | − |
| D-Trehalose | − | + | − | ND | + |

Data for *N. exhalans*, *N. prasina*, *N. metallicus* and *N. listeri* were taken from Yassin *et al*[21], Peltola *et al*.[22] and Schippers *et al*.[23]. +, positive; −, negative; W, weak growth or reaction; ND, no data.

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# Isolation and phylogenetic analysis of one actinomycete strain YIM 90022 exhibiting anticancer activity

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**Abstract**: One facultative alkaliphilic actinomycete strain YIM 90022 was isolated from hypersaline alkaline soil in Qinghai province, China. An almost-complete 16S rRNA gene sequence (1500 bp) for strain YIM 90022 was obtained. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM 90022 was closely related to four members of the genus *Nocardiopsis* with 16S rRNA gene sequence similarity values of 98.8 % (*N. exhalans* DSM 44407$^T$), 98.5% (*N. prasina* DSM 43845$^T$), 98.4% (*N. metallicus* DSM 44598$^T$) and 97.8% (*N. listeri* DSM 40297$^T$), but represented a distinct phylogenetic lineage. Repetitive element sequence-based PCR (rep-PCR) genomic fingerprinting was evaluated on strain YIM 90022 and its closest relatives to investigate their genetic relatedness. The analysis of the rep-PCR genomic fingerprints showed that strain YIM 90022 was distinguishable from its closest relatives. The polyphasic taxonomic data presented in this study, including its morphology, physiological and biochemical characteristics, chemotaxonomy, 16S rRNA gene sequence-based phylogenetic analysis and rep-PCR genomic fingerprinting, supported the view that strain YIM 90022 represented a potential new species of the genus *Nocardiopsis*. The fermentation broth of strain YIM 90022 strongly inhibited growth of cell series of gastric cancer, lung cancer, mammary cancer, melanoma cancer, renal cancer and uterus cancer. Strain YIM 90022 grew well on most tested media, producing exuberant vegetative hyphae and aerial hyphae. The vegetative hyphae are long and fragmented. Light yellow to deep brown diffusible pigments were produced on ISP 2, ISP 3 and ISP 6. Growth of the strain occurred in the pH range 6.0 ~ 12.0, with optimal pH 8.5. The NaCl tolerate range was 0 ~ 15 % (W/V). Cell walls contain meso-diaminopimelic acid and have no diagnostic sugars. Polar lipids are phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanolamine. Major menaquinones are MK-10($H_4$, $H_6$). The DNA G + C content is 71.5 mol %.

**Keywords**: *Nocardiopsis*; Anticancer activity; 16S rRNA gene; Rep-PCR genomic fingerprinting; Phylogenetic analysis

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