Screening and identification of a streptomyces sp.with antifungal activity

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Abstract

[Objective] Screening and identification of a marine-derived actinomycetes with antifugal activity, and then to determine the optimum fermentation conditions. [Method] Candida albicans was used as indicator bacteria to screen active strains. Strain H74-21 was identified based on its morphological, cultural, physiological and sequence analysis of 16S rDNA. HPLC-MS was used to determine the optimal media by analyzing the secondary metabolites. [Results] Strain H74-21 was identified to be a Streptomyces antibioticus strain. The optimum conditions were: 8.0% sucrose, 4.0% yeast extract, calcium carbonate 0.5%, 1L H2O, pH7.0~7.4, 28~30℃, fermentation for 5 days. [Conclusion] A antifungal Streptomyces strain H74-21 was successfully isolated and identified. The optimization of fermentation conditions improved the diversity and total amount of secondary metabolites. This result would be an important reference for further exploring the antifugal secondary metabolites.

Keywords

Streptomyces; Candida albicans; 16S rDNA; H74-21.

1. Introduction

Streptomyces from mangrove sediment is different from the terrestrial streptomyces and the abyssal streptomyces, they grow in the environment with high salinity, low oxygen, less lightness, higher pressure and low nutrition. Thus, mangrove sediment streptomyces have specific genetic material, biological characteristics[1] and metabolites structure types. It is estimated that around 60% of all known antibiotics are derived from secondary metabolites produced by filamentous actinomycete bacteria, most notably Streptomyces species[2].

According to statistics, the mortality rate of Candida infection is about 21% in hospital fungal infection [3]. Antifungal drug such as amphotericin and azole are with severe toxicity, the antifungal spectrum of echinocandin is limited, other antifungal drug appeared drug resistance [4-8]. Therefore, screening and identification of an antifungal streptomyces sp. H74-21 is necessary, the result would be an important reference for further exploring the antifugal secondary metabolites.

1.1 Materials

H74-21 was collected from Yamen Bridge, Xinhui, Guangdong, stored in sand tube. It was provided by the Plant Protection Research Institute, Guangdong Academy of Agricultural Science.

Antifungal indicator bacteria: Candida albicans was purchased from the Guangdong Provincial Food and Drug Administration.

1.2 Screening of the strains

An aqueous medium D, 50 ml in each of four 150-ml Erlenmeyer flasks and was sterilized at 120 ℃ for 30 minutes. A loopful of streptomyces H87-7, H97-7, H21-8 and H74-21, grown on Gause's I medium at 28 ℃ for one week, were inoculated in each of the flasks. The inoculated flasks were

shaken on a rotary shaker (180 rpm) at 28 ℃ for 5 days. The supernatant and mycelium were collected by centrifugation, mycelium was soaked in 95% ethanol for 24h, using disk diffusion method[9] to determine the inhibition zone against Candida albicans. The result suggest only streptomyces H74-21 had antifugal activity.

1.3 Morphology, Cultural , Physiological and Biochemical Characteristics

The cultural characteristics on various agar media were observed after 14 days of incubation at 28 ℃: Gause's I agar, starch agar, potato, Czapek's agar, Kilgler's agar and glucose asparagines agar. The compositions of these media and color descriptions were based on the Streptomyces Identification Manual[11].

Morphological (shown in Figure 1 and Figure 2) and cultural characteristics (shown in Table 1): the color of substrate mycelium was brown, and the color of aerial mycelium was gray. In the electron microscope, aerial hyphae formed spores silk, it was straight and cylindrical.

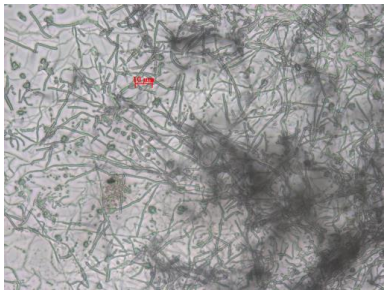

Figure 1.Optical microscope observation of H74-21

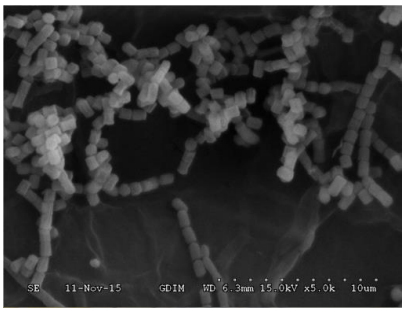

Figure 2.Scanning electron microscopy of H74-21

Table 1. Cultural characteristics of H74-21

Media	Growth	Aerial mycelium Color	Substrate mycelium Color	Shape
Gause's I agar	+++	gray	Ⅲ 76′coffee	Velvet powder, hemispherical
Czapek's agar	+	white	Ⅱ 36′ivory	powder, hemispherical
Kilgler's agar	+++	gray	Ⅰ 67′yellow	powder, hemispherical
Starch agar	+++	grey white	Ⅰ 57′yellow	velvet powder, hemispherical
Glucose aspara-gines agar	++	grey white	Ⅰ 46′yellow	powder, hemispherical
Potato	+++	Ⅰ 11′ivory		powder, hemispherical

Note: "+++" indicates growing very well, "++" indicates growing well, "+" means that can grow. Color contrast to the wallpapers of <Streptomyces Identification Manual>[11].

Testing of gelatin liquefaction, starch hydrolyzate, milk coagulation and peptonization , cellulose utilization, production of H2S and use of carbon source were used to determine the physiological and biochemical characteristics of H74-21. The result suggested that H74-21 had mild ability to make

gelatin liquefaction, strong ability to hydrolyze starch, couldn't grow on cellulose, couldn't make milk coagulation or milk peptonization. It could make use of nine carbon source(shown in Table 2).

Table 2. Carbon source utilization of H74-21

Carbon source	Utilization	Growth
Glucose	+	+
Mannitol	+	+++
Rhamnose	+	++
Sucrose	+	+++
Galactose	+	++
Inositol	+	+++
Arabinose	+	+++
Fructose	+	+++
Xylose	+	++

Note: "+++" indicates growing very well, "++" indicates growing well, "+" means that can grow.

1.4 16S rDNA

Referring Hunfeld's[12] method for extracting of genomic DNA. Universal primers of 16S rRNA gene sequence[13] (forward primer PA: 5'-AGAGTTTGATCC TGGCTCAG-3 '; reverse primer PB: 5'-TACGGTTACCTTGTTACGACTT-3') was used for PCR amplification. PCR amplification products were detected by 1% agarose gel electrophoresis, then sequencing by Shanghai YingJun Biotechnology. At first, use Blast to find related strains of H74-21 from GenBank database, followed by CLUSTALW 1.8 multiple sequence alignment, then use MEGA5.0 software to build phylogenetic tree to determine the genetic relationships and taxonomic status of streptomyces sp. H74-21. 16S rDNA sequences are shown in Figure 3.

GCAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATC
TGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGAGCTGCCCAGGCATCT
GGGTGGCTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAAC
GGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTG
ACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCG
GGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCC
TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCG
ATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGC
CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC
GGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAAC
CCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGT
GAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGT
GCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCA
TGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGG
TGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGG
AGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTT

Figure 3. 16S rDNA sequences of *streptomyces sp.* H74-21

The sequences were placed in GenBank BLAST for similarity analysis, selecting 20 typical strains (Table 3) had high similarity with H74-21 to proceed sequence alignment, most of these strains were

Streptomyces antibiotic and Streptomyces griseoruber, the analysis results are shown in the phylogenetic tree in Figure 4.

Table 3. High similar strains with H74-21 from GenBank

cfcc3080	Streptomyces griseoruber strain cfcc3080
NBRC 12838	Streptomyces antibioticus strain NBRC 12838
CSSP528	Streptomyces antibioticus strain CSSP528
1022-257	Streptomyces antibioticus strain 1022-257
cfcc3075	Streptomyces antibioticus strain cfcc3075
cfcc3085	Streptomyces antibioticus strain cfcc3085
EAAG90	Streptomyces antibioticus strain EAAG90
NBRC 12873	Streptomyces griseoruber strain NBRC 12873
CSSP408	Streptomyces griseoruber strain CSSP408
S5	Actinobacterium S5
15721	Streptomyces bungoensis strain 15721
A316	Streptomyces griseoruber strain A316
HBUM174899	Streptomyces longwoodensis strain HBUM174899
DSM 40089	Streptomyces galbus strain DSM 40089
JM-R35	Streptomyces caeruleatus strain JM-R35
JCM 3373	Streptomyces lasaliensis strain JCM 3373
NBRC 12849	Streptomyces cellostaticus strain NBRC 12849
BCCO 10_1548	Streptomyces curacoi strain BCCO 10_1548
1043	Streptomyces panayensis strain 1043
NBRC 15711	Streptomyces bungoensis strain NBRC 15711

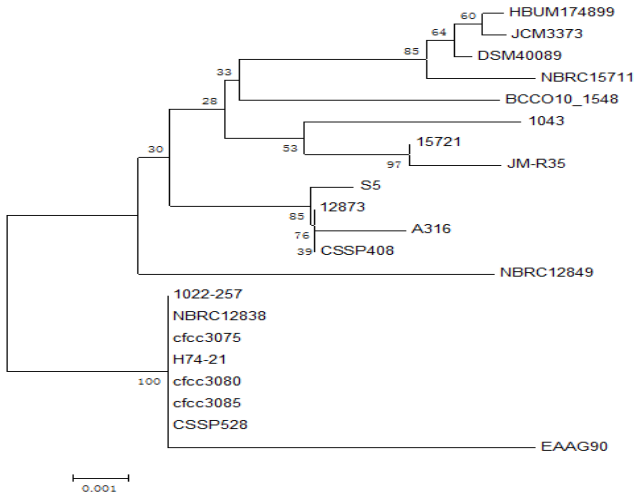

Figure 4. Phylogenetic tree of strain H74-21

1.5 Selection of Fermentation Media

Gause's I medium: soluble starch 20g, potassium nitrate 1g, dipotassium hydrogen phosphate 0.5g, sodium chloride 0.5g, ferrous sulfate 0.01g, magnesium sulfate 0.05g, agar 15g, pH 7.2 ~ 7.4, water 1000mL, pH7.2 ~ 7.4 .

Martin medium: peptone 5g, yeast extract 4g, glucose 20g, dipotassium hydrogen phosphate 0.63g, agar 18g, magnesium sulfate 1.8g, water 1000mL.

Medium A: 8.0% sucrose, 4.0% yeast extract, calcium carbonate 0.5%, water 1000mL, pH 7.0 ~ 7.4.

Medium B: corn starch 2.5%, yeast extract 3.5%, 0.5% seawater crystal, 0.15% calcium carbonate, 0.1% potassium nitrate, 0.06% of magnesium sulfate, 0.09% dipotassium hydrogen phosphate, ferrous sulfate 0.002%, water 1000mL, pH 7.0 to 7.4.

Medium C: 10g starch, 4g peptone, 2g yeast extract, water 1000mL, pH 7.0 ~ 7.4.

Medium D: 3% corn starch, 3% yeast extract, 0.5% seawater crystal, 0.15% calcium carbonate, 0.1% potassium nitrate, 0.06% magnesium sulfate, 0.09% dipotassium hydrogen phosphate, ferrous sulfate 0.002%, water 1000mL, pH 7.0 to 7.4.

Medium E: 2% glucose, 0.5% corn starch, 1% yeast extract, 0.5% peptone, 0.2% sulfuric acid diamine, 0.5% seawater crystal, 0.5% calcium carbonate, dipotassium phosphate 0.1%, water 1000mL, pH 7.0 ~ 7.4.

An aqueous medium A~E, 50 ml in each of five 150-ml Erlenmeyer flasks and was sterilized at 120 ℃ for 30 minutes. A loopful of streptomyces H74-21, grown on Gause's I medium at 28 ℃ for one week, were inoculated in each of the flasks. The inoculated flasks were shaken on a rotary shaker (180 rpm) at 28 ℃ for 5 days. The supernatant and mycelium were collected by centrifugation, mycelium was soaked in 95% ethanol for 24h, HPLC-MS was used to determine the antifugal compounds, the HPLC-MS analysis conditions: 0-20min, 30% -50% A; 20-50min, 30% -50% A; 50-70min, 50% -70% A; 70-100min, 70% A (A is acetonitrile, B was watet with thousandths of formic acid). Type and amount of secondary metabolites of Streptomyces H74-21 were the most after cultivating in media A, the results were shown in Figure 5A~5E, the A media was choosen for fermentation.

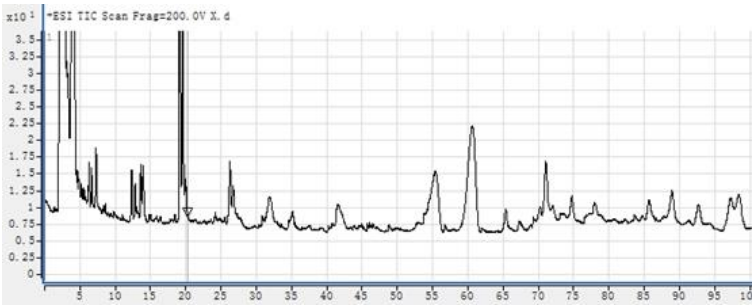

Figure 5A. LC-MS spectra of secondary metabolites of H74-21 fermented by A medium

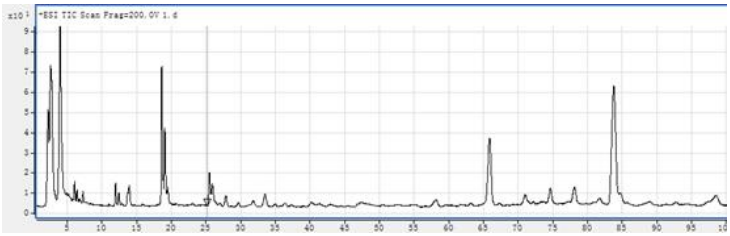

Figure 5B. LC-MS spectra of secondary metabolites of H74-21 fermented by B medium

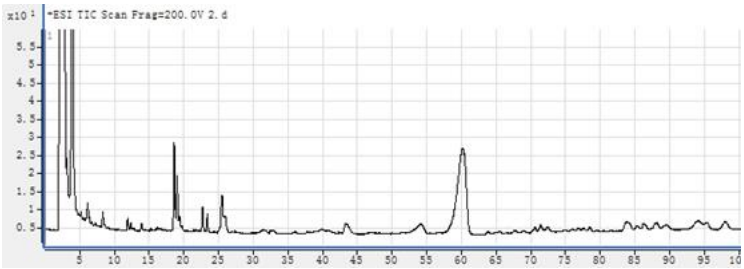

Figure 5C. LC-MS spectra of secondary metabolites of H74-21 fermented by C medium

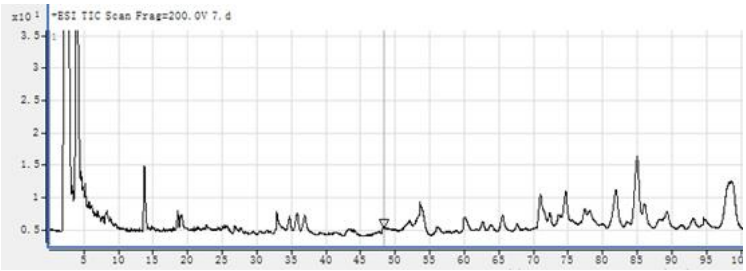

Figure 5D. LC-MS spectra of secondary metabolites of H74-21 fermented by D medium

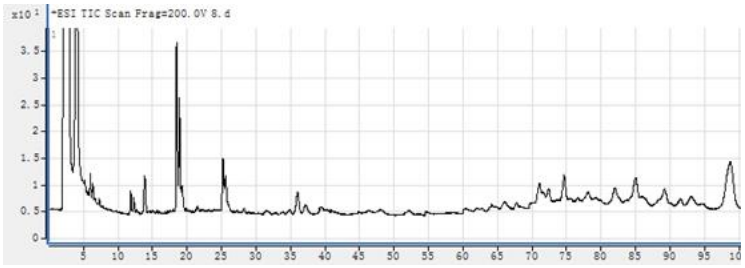

Figure 5E. LC-MS spectra of secondary metabolites of H74-21 fermented by E medium

2. Conclusion

We obtained a marined-derived and antifugal Streptomyces H74-21 by activity screening. H74-21 was fermented on small scale under different culture mediums. Based on the chemical composition analysis to choose the optimal media for fermentation. Strain H74-21 was identified to be a

Streptomyces antibioticus strainbased on its 16S rDNA, as well as morphological characteristics, cultural characteristics, and physiological and biochemical characteristics

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