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PRELIMINARY SCREENING OF SOIL ACTINOMYCETES FOR ANTI-TYROSINASE ACTIVITY

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Short Paper

PRELIMINARY SCREENING OF SOIL ACTINOMYCETES FOR ANTI-TYROSINASE ACTIVITY

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Key words: anti-tyrosinase, screening, *streptomyces.*

ABSTRACT

A bacterial strain TI-B10 was isolated from forest soil in Taiwan. The strain showed 46 U/ml of anti-tyrosinase activity. On the basis of the typical colony appearance and 16S rRNA gene sequences analysis, the strain TI-B10 belonged to the genus *Streptomyces*. The anti-tyrosinase activity of the strain could be improved to 73 U/ml under optimal cultural conditions at pH 8.0 and 30°C in the YMG medium.

INTRODUCTION

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase widely distributed in nature. The enzyme catalyzes the first two reactions of melanin synthesis, the hydroxylation of *L*-tyrosine to 3,4-dihydroxyphenylalanine, *L*-dopa, and the oxidation of *L*-dopa to dopaquinone. This *o*-quinone is a highly reactive compound and can polymerize spontaneously to form melanin [13].

Although the pigment melanin in human skin is a major defense mechanism against the ultraviolet light of the sun, the production of abnormal pigmentation such as melasma, freckles, age-spots, liver spots, and other forms of melanin hyperpigmentation can be a serious aesthetic problem [3]. Hence, inhibiting the tyrosinase activity and preventing the abnormal pigmentations has been the subject of many studies [2, 6, 10-11, 14].

While the search for new tyrosinase inhibitors have been initiated, few reports concerning the screening of microorganisms for anti-tyrosinase activity have been published [9]. Actinomycetes consist of an extensive and diverse group of gram-positive, aerobic, and mycelial bacteria that play an important ecological role in soil cycles. Many are well known for their economic importance as producers of biologically active substances, such as antibiotics, vitamins and other secondary metabolites [1]. The present work reports screening of soil actinomycetes with anti-tyrosinase activity. Both taxonomy and optimal fermentation conditions for the screened strains with anti-tyrosinase activity were investigated.

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MATERIALS AND METHODS

1. Chemicals

Mushroom tyrosinase, *L*-tyrosine, sodium caseinate, asparagine, sodium propionate, nalidixic acid, and cycloheximide were purchased from Sigma (St Louis, MO, USA). Yeast extract, malt extract, glucose and agar were obtained from Difco Laboratories (Detroit, MI, USA). All the materials needed for polymerase chain reaction (PCR) including primers, deoxyribonucleotide triphosphate, *Taq* DNA polymerase were purchased from MDBio (Taipei, Taiwan). Other reagents and solvents used were commercially available and used as received.

2. Screening of soil actinomycetes for anti-tyrosinase activity

A series of 100 soil samples collected over six months in diverse areas of Taiwan were used for isolations of bacterial strains. The fresh soil samples were plated according to the dilution plating method on isolation agar which contained 2 g sodium caseinate, 4 g

sodium propionate, 0.1 g asparagines, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 0.001 g $FeSO_4$, 0.05 g cycloheximide, 0.02 g nalidixic acid, and agar 15 g in one liter distilled water. The pH was adjusted to 7.2 before autoclaving [8].

After cultivation at room temperature for a week, colonies formed on the plates were picked using sterile sticks and transferred to a deep 48-well microplate containing 200 µl of YMG medium that contained 4 g yeast extract, 10 g malt extract, and 4 g glucose in one liter distilled water. The pH was adjusted to 7.2 prior to autoclaving. The microplate was incubated at 180 rpm and 30°C for two days. 60 µl of the primary culture was then transferred to another microplate containing 600 µl of YMG medium for a secondary cultivation. The remaining primary culture was stored at –20°C until the assay of anti-tyrosinase activity was completed. The secondary cultivation was carried out at 180 rpm and 30°C for four days. Then, equal volume of ethanol was added to each well of the secondary cultivation microplate and shaken vigorously for 30 min at room temperature. The cell debris was removed by centrifugation at 4,800 rpm. The supernatant from the extracted broth was assayed to measure the anti-tyrosinase activity.

The modified assay method of anti-tyrosinase activity described in the literature was employed in the present study [9]. 20 µl supernatant was mixed with 80 µl of 0.2 mM *L*-tyrosine dissolved in 50 mM potassium phosphate buffer (pH 6.8). Then, 20 µl of mushroom tyrosinase (1,000 U ml^{-1}, dissolved in the same buffer) was added into each well to initiate the reaction. The assay mixture was incubated at 25°C for 30 min. The increase in absorbance at 475 nm due to the formation of dopachrome was monitored in a microplate reader (microplate reader 2010, Anthos Inc., Salzburg, Austria). The percent inhibition of tyrosinase activity was calculated as follows: % Inhibition = $[(A - B)/A] \times 100$ where A is the absorbance at 475 nm with 50% (v/v) ethanol instead of the tested sample and B is the absorbance at 475 nm with the tested sample.

For confirming the activity of antityrosinase-positive strains, the tested strains were cultivated in a 48-well microplate using YMG. 0.2 ml of the primary culture of each strain was transferred to a 250-ml baffled Erlenmeyer flask containing 20 ml medium and cultivated at 180 rpm and 30°C for 5 days. Samples for biomass and anti-tyrosinase activity assay were collected at predetermined time intervals. Biomass was measured gravimetrically as dry cell weight (DCW) by filtering the sample on a pre-weighed filter paper and drying at 70°C until constant weight. Each sample was serially diluted before the assays of anti-tyrosinase activity. The assay of each dilution was done as described above. The volume of a sample at which 50 % of the enzyme activity was inhibited was obtained by linear curve fitting. One unit of inhibitory activity was defined as the amount of the sample used in the assay condition by which the enzyme activity was reduced to 50%. The anti-tyrosinase activity was expressed as the amount units in one micro liter of broth samples (U/ml).

3. Identification of the strain TI-B10

The strain TI-B10 was identified according to protocol of International Streptomyces Project [ISP, 15]. The strain was cultivated on ISP 2, 3, 4 and 5 media at 30°C for 7 to 14 days. Then, the growth, color of substrate mycelia and aerial mass, sporulation and soluble pigment production of the strain were evaluated.

For determination of the 16S ribosomal RNA gene sequence of the strain TI-B10, chromosomal DNA was isolated as described in the literature [12]. The 16S rRNA gene was amplified using PCR with the forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GGTTACCTTGTTACGACTT-3') primers known to amplify 16S rRNA gene from a broad range of taxonomically different bacterial isolates [7]. PCR was done with a total volume of 100 µl, which contained PCR buffer, 1 µg genomic DNA, 0.2 mM (each) deoxyribonucleotides triphosphate, 50 pmol (each) forward and reverse primers, and 2.5 U of *Taq* DNA polymerase. Amplification was done for 35 cycles in a DNA thermal cycler, Genetic analyzer 377 (Perkin-Elmer, Boston, U.S.A.), employing the thermal profile according to Derakshani *et al*. [7]. The sequence of the amplified DNA fragment was determined by DNA sequencing center of National Cheng Kung University in Tainan. Then, the 16S rRNA gene sequence of the strain TI-B10 was aligned using the BLAST program.

4. Fermentation of the strain TI-B10

The strain TI-B10 was used to prepare a spore suspension. A preserved culture of the strain was inoculated on M207 agar plate which contained 10 g glucose, 20 g soluble starch, 5 g yeast extract, 5 g tryptone, 1 g $CaCO_3$, and 15 g agar in one liter distilled water. The pH was adjusted to 6.8 prior to autoclaving. After incubation at 30°C for a week, 5 ml of sterile water was added to the plate and the spore mass that formed on the aerial mycelium was scraped with an inoculating loop. The spore suspension obtained was transferred to a sterile test tube and stored at –80°C until use.

A seed culture of the strain TI-B10 was initiated by adding 1.5 ml of a thawed spore suspension into a 250-ml baffled Erlenmeyer flask containing 25 ml of YMG medium. The flask was then incubated at 30°C and 180 rpm for 2 days and 2.5 ml of the seed culture was

transferred into each 250-ml baffled Erlenmeyer flasks containing 25 ml of YMG medium for a secondary cultivation. The pHs of the YMG media were adjusted to the desired value (pH 5.0 to pH 9.0) with either 1.0 N sodium hydroxide or sulfuric acid. Then, the culture was carried out at 30°C and 180 rpm for 72 hours. For fermentation at different temperatures, the YMG medium was used and the cultivation was carried out at 180 rpm and the desired temperature (25 to 35°C) for 72 hours. Samples collected at the end of fermentations were assayed as described above. The experiments were carried out in triplicate and the mean values are shown.

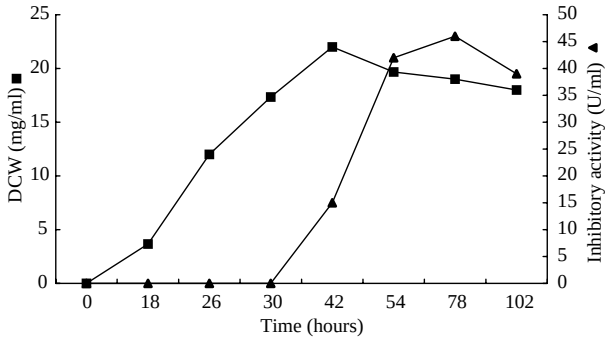

Fig. 1. Cell growth (■) and anti-tyrosinase activity (▲) of TI-B10 grown in shake-flasks at 30°C and 180 rpm. The medium used for strain TI-B10 was YMG medium and its composition was as described in materials and methods.

RESULTS AND DISCUSSION

1. Isolation of strains with anti-tyrosinase activity

Over 10,000 strains were isolated from the 100 soil samples. The screening method was high throughput due to the utilization of 48-well microplates instead of traditional shake flasks in submerged cultivations [5]. Only the first step, which involved transferring the colonies on the agar plates into 48-well microplates with sterile sticks, was performed at one strain per action. All other processes including primary cultivations, secondary cultivations, broth extractions and assays of anti-tyrosinase activity were performed at 8 strains per action with the multichannels pipettes in 48 or 96-well microplates. Using this method, a bacterial strain TI-B10 showed 46 U/ml of anti-tyrosinase activity was isolated (Figure 1).

2. Identification of the strain TI-B10

The cultural characteristics of the strain TI-B10 on various growth media are presented in Table 1. The strain grew well on ISP 2, 3 and 4 media but moderately on ISP 5 medium. The sporulation of the strain was moderate to poor on various ISP media. The color of substrate mycelia varied from brownish orange to red. Aerial mass was pink and switched to orange when it matured. No soluble or melanoid pigment was produced.

A partial 1,404 nucleotides long 16S rRNA gene sequence (not shown) was amplified according to Derakshani *et al*. [7]. Using the BLAST program, it showed the strain TI-B10 was mostly related to members of the genus *Streptomyces*. The highest value of sequence similarity (96.6 %) was obtained between TI-B10 and *Streptomyces sp*. FXJ23 (GI 32307430). Meanwhile, the amplified 16S rRNA gene sequence of the strain TI-B10 was matched over 96.0% with the 16S rRNA gene sequences of another 20 *Streptomyces* species in the data bank. According to the typical colony appearance and 16S rRNA gene sequences analysis, the strain TI-B10 belonged to the genus *Streptomyces*.

3. Fermentation of the strain TI-B10

The strain TI-B10 was cultivated in a serious of different pH and temperatures and analyzed for anti-tyrosinase activity. As shown in Figure 2, the strain TI-B10 exhibited mild difference of anti-tyrosinsae activity between 25 and 35°C but significant difference of activity between pH 5.0 and 9.0. The strain displayed potent anti-tyrosinase activity between pH 7.0 and pH 8.0. However, the strain dramatically lost its activity at

Table 1. Cultural characteristics of the strain TI-B10 on ISP media[a]

Medium	Growth	Substrate mycelia	Aerial mycelia	Sporulation	Soluble pigment
ISP2	Well	Brownish orange	Strong orange	Moderate	None
ISP3	Well	Deep pink	Moderate pink	Poor	None
ISP4	Well	Vivid deep red	Moderate pink	Moderate	None
ISP5	Moderate	Moderate red	Moderate pink	Poor	None

a. ISP 2 to 5 media represent yeast extract-malt extract medium, oatmeal medium, inorganic salt-starch medium and glycerol-asparagine medium, respectively.

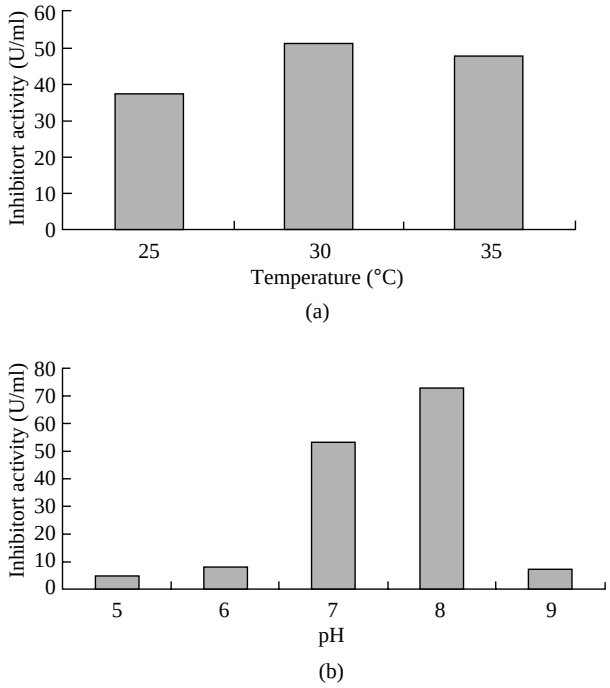

Fig. 2. Inhibitory activity of the strain TI-B10 cultivated at different temperature (a) and pH (b).

pH either lower than pH 6.0 or higher than pH 9.0. The optimal cultural conditions of the strain for anti-tyrosinase activity were at pH 8.0 and 30°C.

In conclusions, the isolated strain TI-B10 displayed 73 U/ml of the anti-tyrosinase activity under optimal cultivation conditions at pH 8.0 and 30°C in the YMG medium. The strain was identified as a species within the genus *Streptomyces* by morphological and 16S rRNA gene sequencing analysis. *Streptomyces* have been known to produce several biologically active substances including antibiotics, vitamins, anti-cancer drugs and other fine chemicals [4]. However, to our knowledge, no tyrosinase inhibitor produced by Streptomyces has been reported until now. Thus, it is worthwhile to purify the active compound from the strain TI-B10 and resolving its structure.

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