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Isolation and characterization of *Streptomyces hiroshimensis* strain TI-C3 with anti-tyrosinase activity

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Synopsis

A bacterial strain, TI-C3, was isolated and verified to display 498 U/ml of anti-tyrosinase activity. Based on morphological, physiological, and chemical analysis, *gyr B* sequences, and DNA-DNA hybridization analysis, the strain TI-C3 was identified as a strain of *Streptomyces hiroshimensis.* The anti-tyrosinase activity of the strain was improved to 905 U/ml under cultivation, using glucose and malt extract as the sole carbon and nitrogen sources, respectively.

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 (1). 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 (2). Hence, inhibiting the tyrosinase activity and preventing the abnormal pigmentations has been the subject of many studies (3–7).

In our laboratory, we were interested in looking for actinomycetes with anti-tyrosinase activity. We used the high-throughput screening method by employing 48-well mi-

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croplates instead of traditional shake flasks in submerged cultivations. Some targets were isolated by this method (8). The present work reports the isolation and characterization of a novel strain, TI-C3, of *Streptomyces hiroshimensis* with the highest anti-tyrosinase activity in our screening study.

MATERIALS AND METHODS

MICROORGANISM

Streptomyces hiroshimensis BCRC 12423 used as an indicator for strain identification was obtained from Bioresources Collection and Research Center, Food Industry Research and Development Institute, Taiwan.

CHEMICALS

Mushroom tyrosinase, L-tyrosine, sodium caseinate, asparagine, sodium propionate, corn steep liquor, maltose, glycerol, sodium nitrate, ammonium sulfate, glucose, nalidixic acid, deoxyribonucleotide triphosphate, and cycloheximide were purchased from Sigma (St Louis, MO). Yeast extract, malt extract, tryptone and agar were obtained from Difco Laboratories (Detroit, MI). *Taq* DNA polymerase needed for polymerase chain reaction (PCR) was purchased from Takara Bio (Shiga, Japan). Primers were purchased from MDBio (Taipei, Taiwan). Other reagents and solvents used were commercially available and used as received.

IDENTIFICATION OF THE STRAIN TI-C3

The strain TI-C3 was identified according to protocol published by Shirling and Gottlieb (9). The melanoid pigment formation was observed on ISP 1, 6, and 7 media. The carbon source utility was determined on basal medium (Pridham-Gottlieb medium) with 1% (w/v) of the tested sugar. The basal medium contained 2.64 g $(NH_4)_2SO_4$, 2.38 g KH_2PO_4, 5.65 g $K_2H_2PO_4 \cdot 3H_2O$, 1.00 g $MgSO_4 \cdot 7H_2O$, 6.4 mg $CuSO_4 \cdot 5H_2O$, 1.1 mg $FeSO_4 \cdot 7H_2O$, 7.9 mg $MnCl_2 \cdot H_2O$, 1.5 g $ZnSO_4 \cdot 7H_2O$, and 18.0 g agar in one liter of distilled water. The pH was adjusted to 6.9 before autoclaving.

The biochemical and physiological characteristics of the strain TI-C3 including growth temperature, melanin production, lysozyme resistance, and substrate hydrolysis were determined by the method of Berd (10). The mycelium of the strain was observed with a light microscope. The spore chain and spore surface morphologies were observed with a scanning electron microscope (Hitachi S-420; Hitachi, Ltd., Tokyo).

Cell wall composition (DL- and LL-diaminopimelic acid isomer, A_2pm) was determined by the method of Hasegawa *et al.* (11). One or two colonies were placed in a cryogenic vial (Evergreen Scientific) with 0.1 ml of 6 N HC1. The vial was autoclaved at 121°C for 15 minutes. After cooling, 1 µl of the hydrolysate was placed on a thin cellulose plate (microcrystalline cellulose; Tokyo Kasei Co., Ltd, Japan). One microliter of 0.01 M DL-A_2pm (Sigma) was spotted on the same plate as a standard. The plate was developed on the solvent system methanol-distilled water-6N HCl-pyridine (80:26:4:10, v/v) for three to four hours. The plate was then dried and sprayed with Ninhydrin spray reagent

(Merck) and heated at 100°C for five minutes. The spots of A_2pm appeared in a yellowish green color.

The sugar content of the cells was analyzed by the same procedure as that of A_2pm, but the hydrolysis and developing were carried out by using 2 N trifluoroacetic acid and n-butanol-distilled water-pyridine-toluene (10:6:6:1, v/v) as solvents, respectively. The spraying reagent was acid aniline phthalate. The standard sugar solution contained 1% (w/v) of each galactose, glucose, mannose, arabinose, xylose, and ribose.

The partial *gyrB* gene fragment was amplified by polymerase chain reaction (PCR) using the primers: PF1 (forward: 5′-GAGGTCGTGCTGACCGTGCTGCACGCGGGCGG-CAAGTTCGGC-3′) and PR2 (reverse: 5′-GTTGATGTGCTGGCCGTCGACGTCGG-CGTCCGCCAT-3′). Genomic DNA was extracted from seven-day cultures using the Qiagen® Genimic DNA Kit. The PCR procedure for amplifying the *gyr B* sequence was the same as that described by Hatano *et al.* (12). The amplified product was analyzed in a genetic analyzer (ABI Prism 310; PE Applied Biosystems, USA) according to the manufacturer's protocol. The *gyr B* sequence of the strain TI-C3 was aligned manually against the nucleotide sequences of other whorl-forming *Streptomyces* strains retrieved from GenBank databases.

DNA-DNA hybridization was performed by the method of Ezaki *et al.* (13). The experiment was performed at least five times, and the level of DNA-DNA hybridization was expressed as the mean percent of the homologous DNA binding value.

FERMENTATION OF THE STRAIN TI-C3

A seed culture of the strain TI-C3 was initiated by adding 1.5 ml of a thawed spore suspension (about 10^8 spores per ml) into a 250-ml baffled Erlenmeyer flask containing 25 ml of YMG medium. The compositions of YMG medium are 4 g yeast extract, 10 g malt extract, and 4 g glucose in one liter of distilled water. The pH of the medium was adjusted to pH 7.2 prior to autoclaving. The flask was then incubated at 30°C for two days at 180 rpm, and 2.5 ml of the seed culture was transferred into a 250-ml baffled Erlenmeyer flask containing 25 ml of modified YMG medium for a secondary cultivation. For fermentation using different carbon sources, glucose in the YMG medium was replaced by the desired carbon source. For fermentation using different nitrogen sources, both yeast extract and malt extract in the YMG medium were replaced by the desired nitrogen source. All cultivations were carried out at 30°C for 72 hours at 180 rpm. After fermentation, an equal volume of ethanol was added to each cultivation and shaken vigorously for 30 minutes at room temperature. The cell debris was removed by centrifugation at 4800 rpm. The supernatant from the extracted broth was assayed to measure the anti-tyrosinase activity. The experiments were carried out in triplicate and the mean values are shown.

ANTI-TYROSINASE ACTIVITY ASSAY

The modified assay method of anti-tyrosinase activity described in the literature was employed in our study (14). Each supernatant sample from the fermentation experiments was serially diluted by a reaction buffer before the assays of anti-tyrosinase activity. Then, 20 µl of each of the diluted samples was mixed with 80 µl of 0.2 mM L-tyrosine

dissolved in 50 mM of potassium phosphate buffer (pH 6.8). Then, 20 µl of mushroom tyrosinase (1000 U ml^{-1}, dissolved in the same buffer) was added to each well to initiate the reaction. The assay mixture was incubated at 25°C for 30 minutes. 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] × 100, where A is the absorbance at 475 nm with reaction buffer instead of the tested sample and B is the absorbance at 475 nm with the tested sample. 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 microliter of broth samples (U/ml).

RESULTS AND DISCUSSION

IDENTIFICATION OF THE STRAIN TI-C3

The screening method used was adopted from our previous work (14) without any modification. Using this method, we screened nearly two thousand isolated strains in a

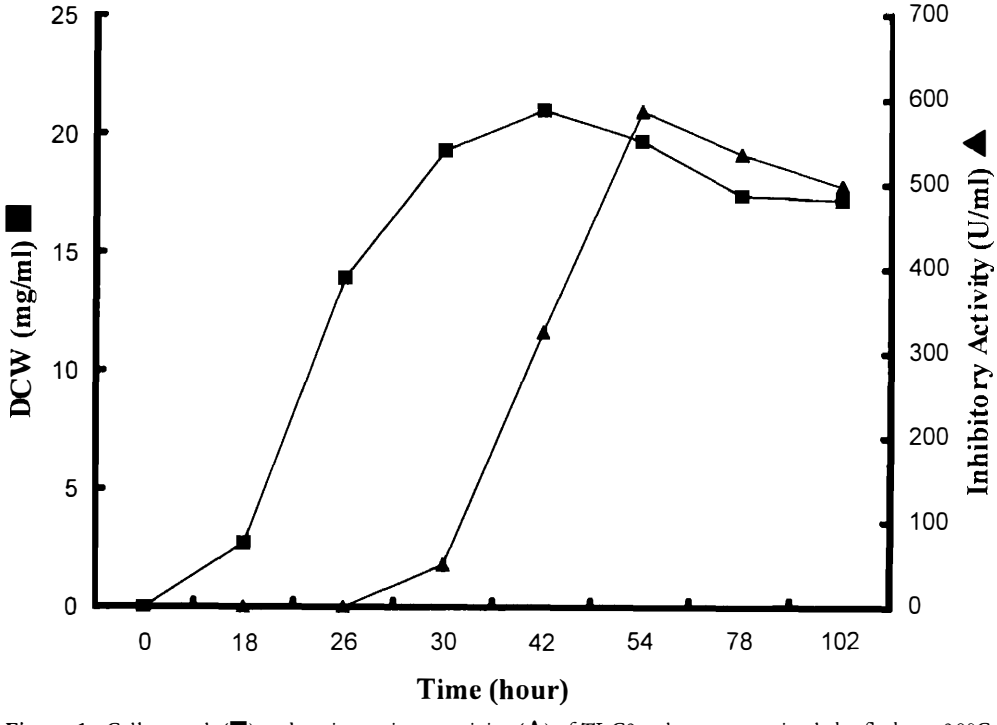

Figure 1. Cell growth (■) and anti-tyrosinase activity (▲) of TI-C3 culture grown in shake flasks at 30°C and 180 rpm. The media used was YMG and its compositions were as described in Materials and Methods. Samples for biomass and anti-tyrosinase activity assay were collected at predetermined time intervals. 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 was attained. The assay of anti-tyrosinase activity was done as described in Materials and Methods.

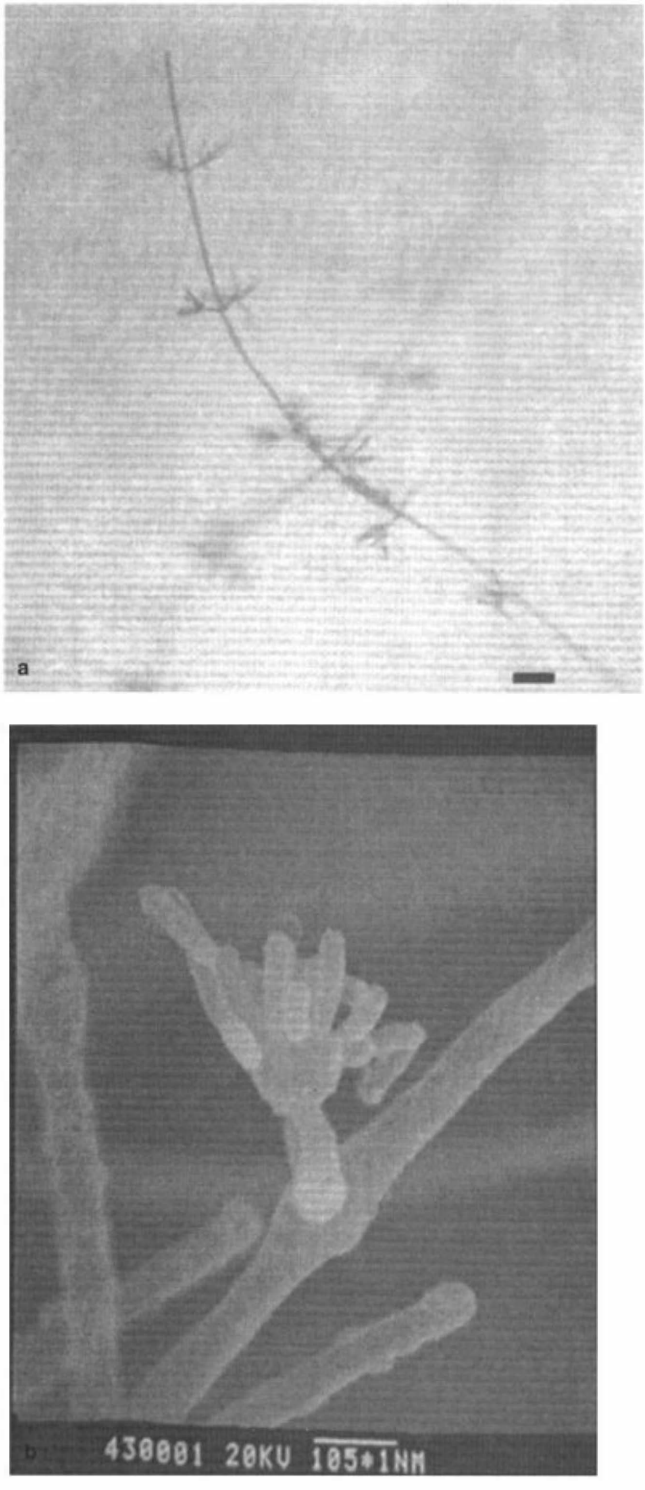

Figure 2. Light (a) and electron (b) micrographs of the strain TI-C3 grown on an oatmeal agar plate at 30°C for 14 days. Bars represent 10 μm and 0.1 μm in the light and electron micrographs, respectively.

month. One strain, named TI-C3, showing 498 U/ml of anti-tyrosinase activity, was isolated from forest soil collected in Taiwan and chosen for further study due to the highest anti-tyrosinase activity (Figure 1).

Under a light microscope, aerial mycelium of the strain TI-C3 exhibited long and straight filaments (Figure 2a). The mycelium also contained spore-bearing branches arranged in whorls at intervals. Sporophores were formed on aerial mycelia, and short umbels of spore chains were observed under a scanning electron microscope (Figure 2b). Analysis of the whole-cell hydrolysate of the strain TI-C3 showed the presence of a chemotype I cell wall characterized by LL-A_2pm. No diagnostic sugars were found. According to the typical colony appearance, microscopic morphologies, and chemical analysis, the strain TI-C3 belonged to the whorl-forming genus *Streptomyces* (formerly *Streptoverticillium*) (15).

For identifying the strain further, a part of sequence (1216 nt) of *gyrB* of TI-C3 was amplified and compared with those whorl-forming strains of *Streptomyces* available from the public databases. The highest value of sequence similarity (97–98%) was observed between TI-C3 and *Streptomyces hiroshimensis* BCRC 12423 (formerly *S. fervens subsp. fervens*). Then, we used DNA-DNA hybridization to identify the homology level between the two strains and obtained a 67.8% homology level. Besides the molecular

Table I
Physiological Characteristics of the Strain TI-C3 Compared with *S. hiroshimensis* Strain BCRC 12423

Characteristics	TI-C3	*S. hiroshimensis* strain BCRC 12423
Growth temperature (°C)	15–40	15–40
Melanin production	–	+
Lysozyme resistance	–	–
Carbon utilization:		
Arabinose	–	–
Cellulose	–	–
Fructose	–	–
Glucose	+	+
Inositol	+	+
Mannitol	–	–
Raffinose	–	–
Salicin	–	–
Starch	–	–
Sucrose	–	–
Xylose	–	–
Substrate hydrolysis:		
Adenine	–	–
Casein	+	+
Esculin	+	–
Gelatin	–	–
Hypoxanthine	+	+
Keratin	–	–
Nitrate	+	+
Tyrosine	+	+
Urea	–	–
Xanthine	–	–
Xylan	–	–

analysis described above, we also identified the strain by using physiological characterizations. Table I lists the comparison of the physiological characteristics between TI-C3 and BCRC 12423. All the criteria except esculin hydrolysis and melanin production gave identical results. Based on these results, the strain TI-C3 was identified as a strain of *S. hiroshimensis.*

FERMENTATION OF *S. HIROSHIMENSIS* STRAIN TI-C3

S. hiroshimensis strain TI-C3 was cultivated using different carbon and nitrogen sources and analyzed for anti-tyrosinase activity. As shown in Figure 3, the strain TI-C3 exhibited maximal anti-tyrosinsae activity (572 U/ml) when glucose was used as the

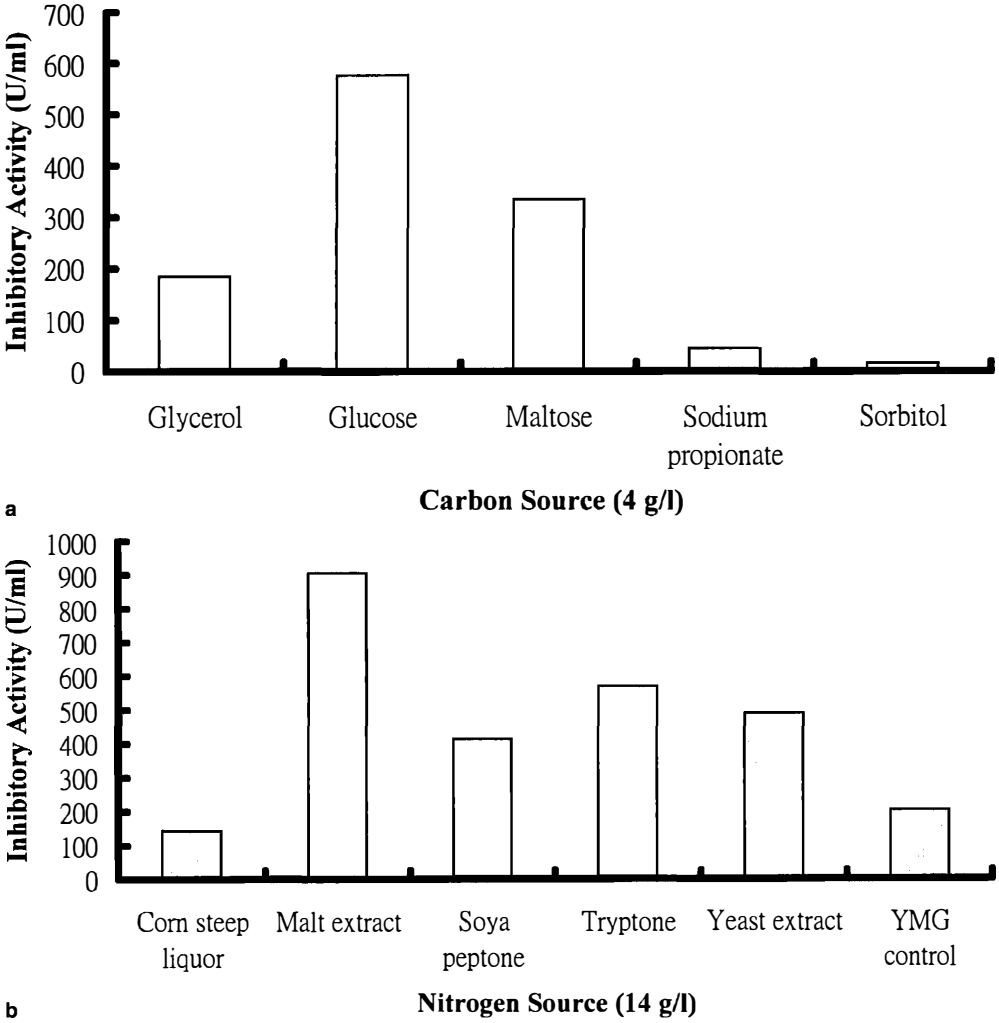

Figure 3. Inhibitory activity of the strain TI-C3 cultivated using different carbon (a) and nitrogen (b) sources. The fermentations and assays of anti-tyrosinase activity were done as described in Materials and Methods.

carbon source (Figure 3a). On the other hand, TI-C3 grew poorly and showed little anti-tyrosinase activity when cultivated using ammonia sulfate, casein, sodium nitrate, or urea as the nitrogen source (not shown). In contrast, TI-C3 displayed potent and mildly different anti-tyrosinase activity when cultivated using more complex and digested nitrogen sources (Figure 3b). TI-C3 showed 905 U/ml of anti-tyrosinase activity when malt extract was used as the nitrogen source.

CONCLUSION

The isolated strain TI-C3 displayed 905 U/ml of the anti-tyrosinase activity under cultivation using glucose and malt extract as the sole carbon and nitrogen sources, respectively. The strain was identified as a strain of *S. hiroshimensis* by morphological, physiological, and chemical analysis, *gyr B* sequences, and DNA-DNA hybridization analysis. The purification of the active compound from the strain TI-C3 is under research in our laboratory.

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