Antioxidant Capacities and Total Phenolic Contents of Selected *Actinomycetes sp.*

นิพนธ์ต้นฉบับ

Original Article

สุพัตรา กุระคำแสง[1], สมพร มูลมั่งมี[2], นงพงา คุณจักร[1] และ ดวงทิพย์ มูลมั่งมี[1]*

[1] ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี กรุงเทพฯ 10140

[2] ศูนย์ความหลากหลายทางชีวภาพ สถาบันวิจัยวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย ปทุมธานี 12120

* **ติดต่อผู้นิพนธ์:** duangtip.moo@kmutt.ac.th

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Supattra Kurakhamsang[1], Somporn Moonmangmee[2], Nongpanga Khunajakr[1] and Duangtip Moonmangmee[1]*

[1] Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, 10140

[2] Biodiversity Research center, Thailand Institute of Scientific and Technological Research, Pathum Thani, 12120

* **Corresponding author:** duangtip.moo@kmutt.ac.th

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บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาฤทธิ์ต้านอนุมูลอิสระและสภาวะที่เหมาะสมต่อการผลิตสารต้านอนุมูลอิสระของเชื้อแอคติโนมัยซีท **วิธีการศึกษา:** เพาะเลี้ยงเชื้อแอคติโนมัยซีท ไอโซเลท SR3.97 ในอาหารเหลว maltose yeast extract (MYEB), tryptone-yeast extract broth (ISP-1) และ Bennett's broth (BN) ที่ความเร็ว 200 รอบต่อนาที ที่ 37 องศาเซลเซียส นาน 7 วัน จากนั้นนำอาหารเลี้ยงเชื้อที่ได้จากการเพาะเลี้ยงเชื้อไปทำแห้งแบบเยือกแข็งและวิเคราะห์ฤทธิ์ต้านอนุมูลอิสระด้วยวิธี 1,1-diphenyl-2-picrylhydrazyl radical-scavenging (DPPH) แล้วเลือกอาหารที่เหมาะสมเพื่อเพาะเลี้ยงเชื้อ และนำตัวอย่างส่วนใสจากการทำแห้งแบบเยือกแข็งมาวิเคราะห์ฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH, 2,2'-azinobis (3-ethylbensothiazoline-6-sulfonic acid) assay (ABTS), Ferric-reducing Antioxidant Power (FRAP) ตรวจวิเคราะห์สารประกอบฟีนอลิกทั้งหมด และศึกษาสภาวะที่เหมาะสมโดยแปรผันค่า pH เริ่มต้นของอาหารเลี้ยงเชื้อที่ 5, 6, 7, 8 และ 9 แปรผันอุณหภูมิเพาะเลี้ยงเชื้อที่ 25, 30, 35, 37, 40 และ 45 องศาเซลเซียส แปรผันระยะเวลาเพาะเลี้ยงเชื้อที่ 1, 3, 5, 7, 9, 11, 13 และ 15 วัน จัดจำแนกเชื้อโดยวิธีทางอนุพันธุศาสตร์ด้วยการตรวจวิเคราะห์ลำดับเบสชิ้นส่วนของยีน 16S rRNA **ผลการศึกษา:** เชื้อแอคติโนมัยซีท ไอโซเลท SR3.97 ผลิตสารต้านอนุมูลอิสระได้ดีที่สุดในอาหาร MYEB และจากวิธี DPPH, ABTS และ FRAP พบว่าสารต้านอนุมูลอิสระที่เชื้อผลิตขึ้นมีฤทธิ์ต้านอนุมูลอิสระได้ต่างกัน (ค่า IC_{50} = 71.52 ± 0.76 µg/ml, 35.88 ± 0.84 µg/ml และ 156.91 ± 1.34 µM Fe(II)/mg ตามลำดับ) มีปริมาณฟีนอลิกทั้งหมดเป็น 57.82 ± 0.1 µg GEA/mg สภาวะที่ผลิตสารต้านอนุมูลอิสระได้สูงสุด คือ ที่ pH เริ่มต้นของอาหารเลี้ยงเชื้อเป็น 7 อุณหภูมิ 37 องศาเซลเซียส และนาน 7 วัน ผลการพิสูจน์เอกลักษณ์พบว่าเชื้อแอคติโนมัยซีท ไอโซเลท SR3.97 คล้ายกับเชื้อ *Streptomyces chrysomallus* subsp. *fumigatus* NBRC 15393^T สายพันธุ์มาตรฐานร้อยละ 99.40 **สรุป:** เชื้อแอคติโนมัยซีท ไอโซเลท SR3.97 ผลิตสารต้านอนุมูลอิสระในอาหารแต่ละชนิดต่างกัน โดยอุณหภูมิ ค่า pH เริ่มต้นของอาหารเลี้ยงเชื้อ และระยะเวลาเพาะเลี้ยง มีผลต่อปริมาณสารต้านอนุมูลอิสระที่ผลิตได้

คำสำคัญ: แอคติโนมัยซีท, สารต้านอนุมูลอิสระ, สารประกอบฟีนอลิก, *Streptomyces chrysomallus*

Abstract

Objective: To investigate antioxidant activity and the optimal condition for antioxidant production from actinomycetes isolate SR3.97. **Method:** Cultivation of actinomycetes isolate SR3.97 was done by inoculation into maltose yeast extract (MYEB), tryptone-yeast extract broth (ISP-1) and Bennett's broth (BN). The cultures were incubated on rotary shaker at 200 rpm, 37 ºC, for 7 days. The culture supernatants were obtained and subsequently lyophilized, and further subject to the analysis of antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl radical-scavenging (DPPH) method. Actinomycetes isolate was cultured in select liquid medium and the lyophilized portion was taken for analysis of antioxidant activity by DPPH, 2,2'-azinobis (3-ethylbensothiazoline-6-sulfonic acid) assay (ABTS), Ferric-reducing Antioxidant Power (FRAP) assay and total phenolic content. Optimal conditions for antioxidant production on initial pH at 4, 5, 6, 7, 8 and 9, temperatures at 25, 30, 35, 37, 40 and 45 ºC and incubation time of 1, 3, 5, 7, 9, 11, 13, 15 days were investigated. Identification of actinomycetes isolate SR3.97 was carried out based on 16S rRNA sequence analysis. **Results:** Actinomycetes isolate SR3.97 was selected as the best isolate for producing antioxidant in MYEB medium. The MYEB medium exhibited antioxidant activity as measured by DPPH, ABTS and FRAP analysis with IC_{50} values of 71.52 ± 0.76 µg/ml, 35.88 ± 0.84 µg/ml and 156.91 ± 1.34 µM Fe(II)/mg, respectively and total phenolic content of 57.82 ± 0.1 µg GEA/mg. The optimal culture conditions for antioxidant production were initial culture pH at 7, incubation temperature of 37ºC, and incubation time for 7 days. Actinomycetes isolate SR3.97 was identified based on 16S rRNA gene sequence analysis. The isolate was 99.40% resembled with *Streptomyces chrysomallus* subsp. *fumigatus* NBRC 15393^T. **Conclusion:** Difference antioxidant levels were produced from actinomycetes isolate SR3.97 when different media were used. The temperature, pH and incubation time affected antioxidant production.

Keywords: Actinomycetes, Antioxidant, Phenolic compound, *Streptomyces chrysomallus*

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Introduction

Actinomycetes is a gram-positive bacterium in the Actinomycetals order. Morphologically, Actinomycetals resembles fungus since it could produce mycelium and spores. Unlike most bacteria, the colony of Actinomycetals is course and not shiny. In agar medium, Actinomycetes produces both subterranean and aerial hyphae. Actinomycetes produces spores with various pigments such as green, orange, brown and black. The habitats of Actinomycetes include soil, animal manure, mud and plants.

Actinomycetes produces primary and secondary compounds including enzymes, anti-microorganism agents, anti-fungals, anti-inflammatory substances, and anti-tumor

agents.[1] Of the 22,500 biologicals, 10,100 of them (45%) are produced by Actinomycetals. Of these 10,100 biologicals, 7,630 are produced by *Streptomyces* sp. and 2,470 by rare actinomycetes.[2] Some of biologicals produced by Actinomycetes have been used as antibiotics such as streptomycin. In addition, Actinomycetes could produce antioxidants such as diphenazithionin which is produced by *Streptomyces griseus* ISP 5236[3] and 5-(2,4-dimethylbenzyl) pyrrolidin-2-one by *Streptomyces* VITSVK5 spp.[4]

Recently antioxidants have received much attention since they could be used for medical, healthcare, and cosmetic purposes, as well as in food industries. Antioxidants, or free-radical scavengers, are compounds that inhibit or slower the oxidative reactions. By giving their hydrogen atoms to free-radical molecules, antioxidants make the free radical molecules more stable, hence not to produce more free-radical molecules and propagate the free radical chain reaction.[5] Since free-radical molecules damage DNA and base sequences, the further synthesis of wrong base sequence could lead to the synthesis of abnormal amino acids.[6] Free radicals produced by lipid peroxidation damage cell membranes, and the functions of proteins, enzymes, and receptor proteins on the cell membranes. With such damages, proteins and enzymes are morphologically abnormal and dysfunctional.[7] Free radicals are also the cause of various diseases such as cancer, heart disease, and arthritis. Well known hyper-reactive free radicals include superoxide anion radicals ($O_2^{-\bullet}$), hydroxyl radicals ($OH^{\bullet}$), oxygen radicals ($1O_2^{-\bullet}$), nitric oxide radicals ($NO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$).[8]

With crucial detrimental health effects of free radical molecules, antioxidants from various sources have been investigated. However, antioxidants from Actinomycetes have been scarcely studied. This study aimed to determine antioxidant activity of the compounds from Actinomycetes, and the conditions optimal for antioxidant synthesis. Findings could be useful in future research on the antioxidant benefits of Actinomycetes.

Materials and Methods

Instruments and reagents

A) Experiment instruments included a multi-mode microplate reader (FLUOstar Omega, BMG LABTECH, Germany), a refrigerated incubator shaker (New Brunswick Innova® 42R/ 43R, Eppendorf AG, Germany), a vacuum freeze dryer (Thermo Savant MicroModulyo Freeze Dryer System, Thermo Electron Corporation, USA), a high speed refrigerated centrifuge (Suprema 25 High Speed Refrigerated Centrifuge, Tomy, Japan), a gel electrophoresis (Cosmo Bio, Bio-active, USA), a cooled CCD imager for gel imaging and documentation (LAS 500, ImageQuant, USA), a PCR thermal cycler (Geneamp PCR system 9700, Applied Biosystems, USA).

B) Chemical reagents were potassium persulfate (Sigma Aldrich), 1,1-diphenyl-2-picrylhydrazyl radical (Fluka), 2,2'-azinobis (3-ethylbensothiazoline-6-sulfonic acid) (Sigma Aldrich), Sodium carbonate (Fluka), gallic acid (Sigma Aldrich), ferric sulfate heptahydrate (Ajax Finechem), Folin-Ciocalteu reagent (Sigma Aldrich), L-ascorbic acid (Wako), ferric chloride hexahydrate (Sigma Aldrich), hydrochloric acid (Merck), 2,4,6-Tris (2-pyridyl)-1,3,5-triazine (TPTZ) (Sigma Aldrich), sodium acetate (Fluka), agarose (Bio-Active ISC Bio express), and absolute ethanol (Merck).

C) Culture broth consisted of yeast extracts (BD), malt extracts (Merck), dextrose (Difco), agar (BD), maltose (Ajax Finechem), beef extract (BD), glucose (Merck) and casein enzymatic hydrolysate (Sigma Aldrich).

D) Genomic DNA sequence analysis consisted of DNA extraction kit (GF-1 kit, Vivantis), PCR amplification kit (Takara Taq, Takala), 27f and 1525r primers (Bio-Rad Laboratories, Inc.), PCR product purification kit (QIAquick PCR Purification, Qiagen)

Experimental procedure

A) Development of starter culture

Actinomycetes isolate SR3.97 was generated from a sample of termite mound soil as a courtesy of Sakaerat Environmental Research Station (Nakhonratchasima). The cultivation of actinomycetes isolate SR3.97 was done by inoculation in ISP-2 hard broth.[9] The cultures were incubated on at 37 ºC, for 7 days. Actinomycetes was allowed to grow and generate spores in the culture broths. Cork-borer (0.5-cm diameter) was used to cut broth agar of the starter. Each of the twenty pieces of the starter were transferred into 50-mL liquid broth in a 250-mL Erlenmeyer flask for further antioxidant activity determination.

B) Cultivation of Actinomycetes in various liquid broths

The starter culture of actinomycetes mentioned above was transferred into three liquid broths including maltose yeast extract (MYEB), tryptone-yeast extract broth (ISP-1)[10] and Bennett's broth (BN)[11] with a transfer volume of 50 mL in a 250-mL Erlenmeyer flask. With an initial broth pH of 7.2 ± 0.2, the cultures were incubated on rotary shaker at 200 rpm, 37 ºC, for 7 days.

C) Preparations of lyophilized Actinomycetes samples

Actinmycetes culture was separated from the broth by centrifugation with 7,000 rpm for 10 minutes. The culture supernatants were obtained and subsequently lyophilized and kept at -20 ºC prior to analysis.

D) Analysis of antioxidant activity and phenolic content

Antioxidant activity of Actinomycetes culture was determined using three methods namely DPPH radical scavenging assay, 2,2'-azinobis (3-ethylbensothiazoline-6-sulfonic acid) (ABTS) assay, and Ferric-reducing Antioxidant Power (FRAP) assay.

DPPH radical scavenging assay quantifies the ability of a substance in giving hydrogen atom to free radicals. As a free radical molecule, DPPH solution is purple. Once receiving a hydrogen atom from an antioxidant, the reaction mixture turns from purple to yellow. This method was modified from that of Hatano et al (1989).[12] The lyophilized samples of Actinomycetes isolate SR3.97 were dissolved in distilled water to reach final concentrations of 20 – 100 microgram per milliliter. A volume of 100 µL of the sample was pipetted and transferred into each of the 96-well microplate. A volume of 100 µL of 100 µM DPPH in absolute ethanol was added in the microplate well. After incubating in the dark at room temperature for 30 minutes, the absorbance of DPPH of each sample was determined at 517 nm relative to a control (i.e., ascorbic acid as a standard antioxidant) on the multi-mode microplate reader with 3 replicates. The % inhibition on DPPH was calculated as follows:

$$\% \text{ Inhibition} = [(Abs_{control}-Abs_{sample})/Abs_{control}] \times 100$$

where A_{sample} and $A_{control}$ were the absorbance of DPPH in the resultant reaction mixture, i.e., test sample, and control. The equation between % DPPH inhibition and concentrations of antioxidant from Actinomycetes isolate SR3. 97 was determined so that the 50% DPPH inhibitory concentration (IC_{50}) of the Actinomycetes isolate SR3. 97 could be further determined.

The 2,2'-azinobis (3-ethylbensothiazoline-6-sulfonic acid) (ABTS) assay also measures degree of hydrogen atom that study compound gives to a free radical. Since ABTS is stable and not a free radical, it needs to be transformed into a free radical by oxidation caused by potassium sulfate to a blue-green solution. Once ABTS free radical is inhibited by an antioxidant, the solution turns from blue-green to green, and finally colorless, depending on the hydrogen releasing ability of the antioxidant. This ABTS assay was modified from that of Re et al. (1999).[13]

ABTS solution in distilled water with a concentration of 7 mM was prepared. The ABTS solution was mixed with the 140 mM potassium persulfate solution in a ratio of 5 mL to 88 mL, and incubated in the dark for 12 – 16 hours. The resultant ABTS free radical ($ABTS^{\bullet+}$) with green-dark blue color was obtained. This solution was then diluted with distilled water and measured for absorbance at 734 nm. The absorbance of 0.700 ± 0.2 was obtained before the test sample's absorbance could be measured.

The lyophilized samples of Actinomycetes isolate SR3.97 were dissolved in distilled water to reach final concentrations of 20 – 100 microgram per milliliter. Ascorbic acid solutions with concentrations ranging from 2 – 10 µg per mL were prepared. 20 µL sample solution of various concentrations and 180 µL ABTS solution were added into each well of the 96-wells microplate. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance at 734 nm was measured on the multi-mode microplate reader with 3 replicates. The % inhibition on ABTS was calculated as follows:

$$\% \text{ Inhibition} = [(Abs_{control}-Abs_{sample})/Abs_{control}] \times 100$$

where A_{sample} and $A_{control}$ were the absorbance of ABTS in the resultant reaction mixture, i.e., test sample, and control. The equation between % ABTS inhibition and concentrations of antioxidant from Actinomycetes isolate SR3. 97 was determined so that the 50% ABTS inhibitory concentration (IC_{50}) of the Actinomycetes isolate SR3. 97 could be further determined.

Lastly, the **Ferric-reducing Antioxidant Power (FRAP) assay** determines antioxidant activity by measuring reduction or electron donating capacity of the antioxidant to the free

radical which is FRAP reagent. As a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex, once receiving an electron from an antioxidant, FRAP reagent turns into ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex which is a stable compound with purple-blue. The color is darkened with the increasing electron donating capacity of the antioxidant. This method was modified from that of Kong et al. (2015).[14]

FRAP solution was prepared from three solutions in 40 mM hydrochloric acid, namely 3 mM acetate buffer solution (pH 3.6), 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$), and 10 mM 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ) in a ratio of 10:1:1 (v/v). To protect from light, this yellow solution was stored in an ample bottle.

The lyophilized samples of Actinomycetes isolate SR3.97 were dissolved in distilled water to reach final concentrations of 200, 500 and 1,000 µg per mL. 20 µL sample solution and 180 µL FRAP solution were transferred into each well of the 96-wells microplate. The mixture was incubated in the dark at room temperature for 30 minutes. Absorbance at 593 nm was measured on the multi-mode microplate reader with 3 replicates. Relative antioxidant activity (FRASP value in µM Fe(II)/mg was obtained from the curve of absorbance against concentrations of ferric sulfate heptahydrate ($FeSO_4.7H_2O$), with final concentrations of 2 – 6 mM.

E) Analysis of the total phenolic content

Phenolic compounds react with Folin-Ciocalteu reagent to form a blue mixture. Folin-Ciocalteu reagent contains phosphomolybdic-phosphotongstic acid which could be reduced by hydroxyl group of phenolic compounds to form tungsten and molybdenum blue. This resultant substance has a blue color. The Folin-Ciocalteu method to quantify total phenolic content was modified from that of Odenh et al. (2014).[15] The solution of Folin-Ciocalteu reagent in distilled water (1:9, v/v) and the solution of sodium carbonate (7.5% w/v) were prepared. isolate SR3.97

The lyophilized samples of Actinomycetes isolate SR3.97 were dissolved in distilled water to reach final concentrations of 200 – 1,000 µg per mL. Gallic acid solutions as the standard with concentrations of 4 – 12 µg per mL were prepared. 20 µL of sample solution and standard solution were transferred into each well of the 96-wells microplate. 100 µL Folin-Ciocalteu solution and 80 µL 7.5% sodium carbonate solution were added into each well. The mixture was incubated at room temperature for one hour. Absorbance at 765 nm was measured on the multi-mode microplate reader with 3 replicates. Total phenolic content was presented as gallic acid equivalent (GAE) per 1 gram dry weight sample (µg GAE/mg dry weight sample).

F) Optimal conditions for antioxidant synthesis by Actinomycetes

Once type of broth that promoted the highest antioxidant synthesis was determined by DPPH assay, that broth was further used in determining the optimal conditions for antioxidant synthesis. The procedures of starter culture (A), cultivation of Actinomycetes in various liquid broths (B), and preparations of lyophilized Actinomycetes samples (C) were repeated. The conditions studied were as initial broth pH, temperature, and duration of cultivation.

To study effects of **initial broth pH**, initial pH of 5, 6, 7, 8 and 9 were tested. The cultures were incubated at 37 ºC, on rotary shaker at 200 rpm for 7 days. Actinomycetes isolate SR3.97 was obtained and analyzed for antioxidant activity by DPPH assay as mentioned above.

For **cultivation temperature**, once optimal pH was determined, that pH was used to further determine optimal temperature. The incubation was conducted at 25, 30, 35, 37, 40 and 45 ºC on rotary shaker at 200 rpm for 7 days. Actinomycetes isolate SR3.97 was obtained and analyzed for antioxidant activity by DPPH assay as mentioned above.

In terms of **cultivation duration**, the longest duration of 15 days on rotary shaker at 200 rpm was tested with sampling times at 1, 3, 5, 7, 9, 11, 13 and 15 days. The procedure was based on the optimal initial broth pH and temperature previously determined. Actinomycetes isolate SR3.97 was obtained and analyzed for antioxidant activity by DPPH assay as mentioned above.

G) Identification of Actinomycetes isolate SR3.97

To identify Actinomycetes isolate SR3.97, base sequences of 16S rRNA were identified. Actinomycetes isolate SR3.97 was inoculated ISP-2 hard broth for 2 days. Genomic DNA of the isolate was extracted by GF-1 kit (Vivantis) and complete 16S rRNA gene was multiplied by PCR using the PCR Amplification Kit with Takara Taq (Takala) with universal primer including 27F primer (5' AGA GTT TGA TCC TGG CTC AG 3') and 1525 R primer (5' AAA GGA GGT GAT CCA GCC 3').[16] PCR product was purified by the QIAquick

PCR Purification Kit (Qiagen) and analyzed for nucleotide sequences by direct sequencing provide by Word Medic Ltd.. Base sequences were further trimmed and edited using ChromasPro software program version 1.7.7 and compared with standards by homology search using the standard BLAST sequence similarity searching in the EzTaxon server (http://www.ezbiocloud.net/eztaxon/identify) against previously reported sequence at the GenBank/ EMBL/ DDBJ database.[17] Multiple alignment of edited sequencing was conducted at the GenBank/ EMBL/ DDBJ using ClustalW multiple alignment software program.[18] Verified and adjusted alignment was used for building phylogenetic tree based on the neighbor-joining[19] in the Molecular Evolutionary Genetics using the Analysis (MEGA) software version 6. Confidence level of association was obtained based on 1,000 bootstrap replicates.

Results

After the cultures of Actinomycetes isolate SR3.97 were incubated on rotary shaker at 200 rpm, 37 ºC, for 7 days, the culture supernatants were freeze dried by lyophilization. It was found that among MYEB, BN and ISP-1 liquid broths, DPPH analysis revealed that MYEB broth allowed Actinomycetes isolate SR3.97 to produce the highest antioxidant activity (68.11 ± 0.68 µg/ml), followed by BN and ISP-1 broths (31.14 ± 0.96 and 7.83 ± 0.81 µg/ml, respectively). As a result, MYEB broth was selected for determining optimal condition (Figure 1).

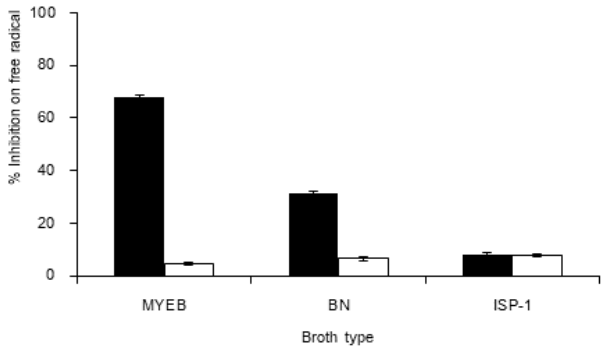

Figure 1 % Inhibition on free radical by DPPH method in MYEB, BN and ISP-1 broth inoculated with Actinomycetes isolate SR3.97(■) compared with that with no inoculation (□) in the conditions of initial pH of 7.2 ± 0.2, 200 rpm, for 7 days.

In terms of assay methods, **antioxidant activity** of Actinomycetes isolate SR3.97 as presented by IC_{50} was found to be 71.52 ± 0.76 µg/ml by DPPH assay, and 35.88 ± 0.84 µg/ml by ABTS assay (Table 1). Antioxidant activity as presented by electron donation activity by FRAP method was 156.91 ± 1.34 µM Fe(II)/mg. In addition, MYEB with no cultivation (negative control) was found to have antioxidant activity lower than MYEB with cultivation (culture supernatant). Higher antioxidant activity was found in ascorbic acid (positive control) by DPPH and ABTS methods since its IC_{50} was lower than those of the Actinomycetes isolates. Ascorbic acid also provided a higher level of electron donation activity than the isolates (Table 1).

Table 1 Antioxidant activity of Actinomycetes isolate SR3.97 by DPPH, ABTS and FRAP methods with the inoculated MYEB broth with an initial pH of 7.2 ± 0.2, with an oscillation of 200 rpm, 37 °C for 7 days.

Samples	IC_{50} of DPPH (µg/mg)	IC_{50} of ABTS (µg/mg)	FRAP value µM Fe(II) /mg)
Inoculated MYEB broth	71.52 ± 0.76	35.88 ± 0.84	156.91 ± 1.34
MYEB broth (negative control)	6048.95 ± 0.47	582.68 ± 0.57	8.07 ± 1.62
Ascorbic acid (positive control)	3.78 ± 0.52	3.81 ± 0.04	12,414.52 ± 0.04

Regarding **total phenolic content**, the highest level was found in Actinomycetes isolate SR3.97 cultivated in MYEB broth (57.82 ± 0.12 µg GEA/mg). This was higher than the one from the MYEB broth with no cultivation (negative control), which was 4.04 ± 0.32 µg GEA/mg.

In determining the **optimal conditions** for producing compounds with antioxidant activity, **initial broth pH** of 7 resulted in the highest antioxidant activity with 76.91% DPPH inhibition and a related IC_{50} of 71.65 ± 0.64 µg/ml. Antioxidant activities from Actinomycetes isolates (100 µg/mL) cultivated in broths with initial pH of 5, 6, and 8 were lower than that cultivated in broth with initial pH of 7 (Figure 2). For optimal **cultivation temperature**, the optimal temperature was 37 ºC where DPPH inhibition was 78% with an IC_{50} of 71.35 ± 0.83 µg/ml. On the other hand, cultivation at 25, 30 and 35 ºC resulted in DPPH inhibitions of less than 50%. In addition, cultivation at 40 and 45 ºC offered the lowest antioxidant activity (Figure 3). In terms of **cultivation duration**, isolates (100 µg/mL) from the cultivation of 5 – 7 days resulted the highest antioxidant activity with % DPPH inhibition of 60 – 71% with IC_{50} values in a range of 71 – 79 µg/ml. Specifically, isolate with 7 days of cultivation provided the highest antioxidant activity with an IC_{50} of % 71.13 ± 0.38 µg/ml. After

7 days till the 15 days of cultivation, the antioxidant activity decreased (Figure 4).

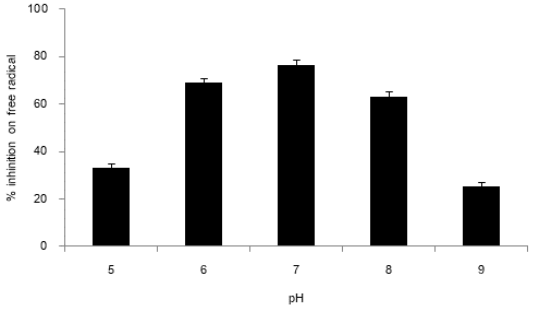

Figure 2 % Inhibition on free radical by DPPH method in MYEB inoculated with Actinomycetes isolate SR3. 97 with various pH, 200 rpm, 37 °C, for 7 days.

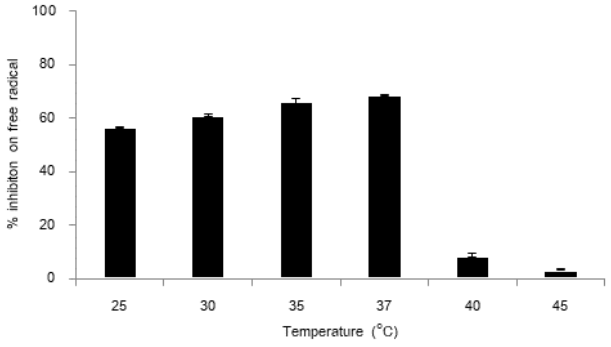

Figure 3 % Inhibition on free radical by DPPH method in MYEB inoculated with Actinomycetes isolate SR3.97 with pH 7, 200 rpm, various temperature, for 7 days.

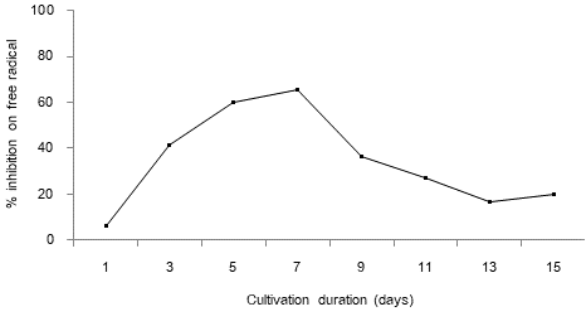

Figure 4 % Inhibition on free radical by DPPH method in MYEB inoculated with Actinomycetes isolate SR3.97 with pH 7, 200 rpm, 37 °C, for various cultivation durations.

In terms of identification of Actinomycetes isolate SR3.97, base sequence analysis of 16S rRNA revealed that Actinomycetes isolate SR3.97 had the base sequence 99.40% similar to that of 16S rRNA of standard strain of *Streptomyces chrysomallus* subsp. *fumigatus* NBRC 15394^{T}. The evolution of Actinomycetes isolate SR3.97 as phylogenetic tree produced by Neighbor-joining model is shown in Figure 5.

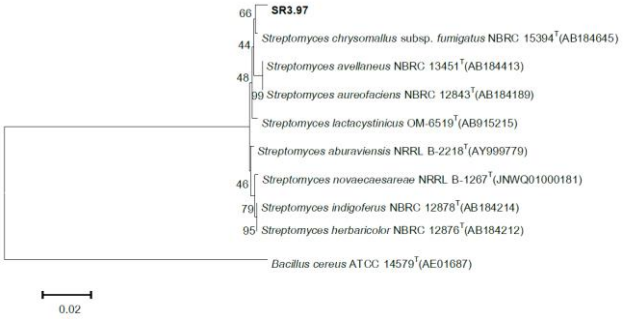

Figure 5 Phylogenetic tree based on the base sequence analysis of Actinomycetes isolate SR3.97 compared the 16S rRNA of standard strain of *Streptomyces chrysomallus* subsp. *fumigatus* NBRC 15394^{T}.

Discussions and Conclusion

In this present study, Actinomycetes isolate SR3.97 was selected to determine the production of compounds with antioxidant activity in three different kinds of liquid broths including MYEB, BN and ISP-1. Actinomycetes isolate SR3.97 could produce and accumulate compounds with antioxidant activity in all three kinds of broth. Since MYEB provided the highest antioxidant activity, this best should be further studied with Actinomycetes isolate SR3.97. This discrepancy was due to the differences in kind and amount of carbon and nitrogen in these broths. In MYEB, carbon source is from maltose; while BN has glucose and ISP-1 has casein enzymatic hydrolysate as the source of carbon. Different sources of carbon usually lead to different influx of nutrients to the cell for compound synthesis.

In terms of nitrogen source, MYEB and BN broths provide similar nitrogen source (beef extract and yeast extract) but with different amounts. ISP-1, on the other hand, contains yeast extract as a nitrogen source with an amount different from the other two broths. A higher level of nitrogen could cause stress which in turn allow less nutrients into cells. In addition, source with less nitrogen could result in less nutrients for the cell to produce antioxidant compounds.

Our study found that MYEB broth provided optimal nutrients for producing antioxidant compounds in Actinomycetes isolate SR3.97. This is because MYEB contains maltose as carbon source (10 gm per liter), and

appropriate source of nitrogen from yeast extract and beef extract (2 and 1 gm per liter, respectively). Our finding was consistent with a study reporting that amount and kind of carbon and nitrogen source affected antioxidant producing capacity of bacterial isolates.[11]

In MYEB broth, antioxidant activities of Actinomycetes isolate SR3.97 by DPPH, ABTS and FRAP methods were different with IC_{50} of the first two methods of 71.52 ± 0.76 and 35.88 ± 0.84 µg/ml, respectively, and antioxidant activity as presented by electron donation activity by FRAP method of 156.91 ± 1.34 µM Fe(II)/mg. This discrepancy was due to different reactions and limitations among assay methods, and molecular size of the antioxidants. These factors affect the capacities of antioxidant molecules in reacting with free radicals. Therefore, multiple assay methods, rather than single method, are recommended in antioxidant activity study. However, the method most optimal for given antioxidants should be determined.

In general, DPPH assay has been the most used method for antioxidant activity quantification. This is because it is convenient, fast, applicable both for water and ethanol soluble substances. It is also able to quantify antioxidant activity of phenolic compounds. In our study, the isolate contained a large amount of phenolic compounds and was water and ethanol soluble. The characteristics of the isolate was applicable for DPPH assay. In addition, since DPPH structure, as a free radical, has three benzene rings, large-molecule antioxidants could sometimes have difficulties to provide the electron to the uncoupled single electron of DPPH. As a result, antioxidant activity could less prominent by DPPH assay.[20]

ABTS assay has been applicable for all water-soluble antioxidants.[21] Even though ABTS contains benzene ring like DPPH, its uncoupled singled electron is not obstructed by the benzene ring. As a result, more antioxidants in isolates could react more free radicals, thus exert more antioxidant activity. FRAP assay depends on reduction or electron donation capacity of the antioxidants to free radicals in acidic environment. Since mechanism of FRAP with antioxidants are different from those of DPPH and ABTS, antioxidant activity measured by FRAP is obviously different from the first two methods.

Even though they produced different antioxidant activity results, once compared with negative control and ascorbic acid as positive control, the three assays provided comparable results. Lyophilized Actinomycetes isolates SR3.97 showed antioxidant activity higher than that of the negative control, and lower than that of ascorbic acid as the positive control. This finding was similar with the three assays. In general, for a given set of conditions, antioxidant activity levels by the three methods were unequal because of reaction mechanisms and structures of free radicals and antioxidants were different.

The results of total phenolic content were consistent with those of the antioxidant activity by DPPH assay. This is because DPPH assay is one of the methods to quantify antioxidant activity of phenolic compounds. Our results confirmed other previous reports that most phenolic compounds exert antioxidant activity.[11] Therefore, certain portion of antioxidants found in the Actinomycetes isolate SR3.97 was phenolic compounds.

For optimal conditions for producing antioxidants, initial acid-base environment of the broth, temperature and cultivation duration were found to affect antioxidant producing capacities of the Actinomycetes isolate SR3.97. This was due to the fact that pH and temperature affect the cell structure and the transportation of nutrients into cells. They could also lead to dysfunctions of receptors on cell membrane. These could lead to abnormal capacity to produce antioxidants.[22] In terms of cultivation duration, it was found that the Actinomycetes could produce antioxidants slowly in the first 1 to 3 days of cultivation. This is because Actinomycetes grows slowly. Antioxidants which are secondary metabolites are therefore slowly produced in the early phase of their growth. The production of antioxidant was increased to reach the peak at day 7. The production then declined through day 15. This declining phase could be due to a gradual accumulation of wastes by the microorganism. In addition, the microorganism could produce certain compounds such as hydroxypipecolic acids which could damage antioxidant structure hence decrease their antioxidant activity.[23]

The identification of Actinomycetes isolate SR3.97 by nucleotide sequence analysis revealed that its 16S rRNA was 99.40% similar that of *Streptomyces chrysomallus* subsp. *fumigatus* NBRC 15394^T. Since *Streptomyces* sp. has been scarcely studied for antioxidant producing capacity, there should more research to determine biological effects of Actinomycetes especially antioxidant activity, purification, cell toxicity, anti-tumor effect and anti-inflammatory effect. The information could be useful in medical, cosmetic, pharmaceutical as well as food industries.

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