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Full Length Research Paper

The *in vitro* antioxidant properties of *Bacillus simplex* XJ-25 isolated from sand biological soil crusts

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XJ-25, a strain with strong antioxidant activity was isolated from sand biological soil crusts in Gurban Tonggut Desert, Xinjiang, China. Strain XJ-25 is closely related to *Bacillus simplex* through the 16s rDNA sequencing combined with morphological, physiological and biochemical analysis. The medium for optimal antioxidant activity was NB with 1.5 g/L glucose. Based on the kinetic assay, antioxidant activity began at early exponential growth phases; maximum activity was reached at the stationary phase. Scavenging effects on 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), the hydroxyl and superoxide radicals, T-AOC capacity and protection against lipid damage were evaluated. The main antioxidant compounds were in the extracellular secreted supernatant of XJ-25. The active compounds were very stable at a pH range of 2 to 12, and temperature from 40 to 121°C, as well as in some organic solvents. Thin layer chromatography assay by DPPH scavenging assay showed two active spots with a Rf values of 0.35 and 0.47, and both of them were ninhydrin positive.

Key words: Antioxidant activity, *Bacillus simplex*, TLC, bacteria, sand soil biological crusts.

INTRODUCTION

It is well known that free radicals can damage deoxyribonuleic acid (DNA), proteins, lipids and carbohydrates within the human tissue and cause many diseases, such as cancer, aging and diabetes (Baskar et al., 2004; Meghashri et al., 2010; Halliwell, 1991). Active antioxidant compounds can scavenge free radicals and reactive oxygen species (ROS), and also prevent the generation of free radicals and ROS, and/or activate a battery of detoxifying proteins to delay or prevent the oxidation of cellular oxidisable substrates. Recently, widely used synthetic antioxidants, such as butylhydroxyanisole (BHA), butylhydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ) are being questioned for their probable toxic and carcinogenic effects (Grice, 1986; Wichi, 1988). Therefore, the development and utilization of more effective and less harmful antioxidants of natural origins are desirable. Alternatively, microbial sources have been shown to be a powerful potential source producing many kinds of naturally functioning products.

Sand biological soil crusts (BSCs) under extremely drought and strong UV irradiation stressful environment are a unique mini-nature landscape in desert areas, as well as the obvious sign of fixing mobile dune (Gundlapally and Garcia-Pichel, 2006). And sand biological soil crusts at extreme environment are suffering with extremely drought and strong UV irradiation. It is considered that extreme desiccation and irradiation can increase the formation of reactive oxygen species in organisms, and bacteria are highly resistant to this kind of potential damage (He and Der, 2002). To prevent sand biological soil crusts from the damage of oxidative stress caused by drought and irradiation, the bacteria from sand BSCs may contain considerable amount of antioxidant substances.

There were not many reports about bacteria producing active antioxidant compounds lately. Bacteria isolated from sand BSCs and its antioxidant activity have not been demonstrated. Therefore, our work is attempting to isolate bacteria producing active antioxidant compound from sand BSCs, production, and to investigated the

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biological and biochemical properties of the antioxidant activity produced by XJ-25.

MATERIALS AND METHODS

Bacterial strain

Bacillus simplex XJ-25 was originally isolated from BSCs collected in Gurban Tonggut desert, Xinjiang China. It was maintained on agar slants containing (g/L): Glucose 1.5 g; nutrient agar 3.8 g. The slants were incubated at 35°C, for 24 h and the fully grown slants were stored at 4°C.

Sample preparation

B. simplex XJ-25 strain was grown in 100 ml of nutrient broth with glucose (1.5 g/L) at 28°C with constant shaking at 170 rpm. Fermentation broths were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant of extracellular secretion was filtered through a 0.22 µm membranes. For the preparation of intracellular cell-free extracts, cell pellets were washed twice with deionized water and resuspended in deionized water followed by ultrasonic disruption. Sonication was performed for five 1-min intervals in an ice bath. Cell debris was removed by centrifugation at 10,000 rpm for 15 min, and the supernatant was the intracellular cell-free extract. Total cell numbers were adjusted to 10^9 CFU/ml for the preparation of intracellular cell-free extracts. The extracellular substance and the intracellular cell-free extract were measured for the antioxidant activity, respectively.

Isolation and identification of XJ-25

XJ-25 was identified by 16S rDNA sequencing analysis with polymerase chain reaction (PCR) using the universal primers, 27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R(5'-GGT TACCTTGTTACGACTT-3'). Sequence analysis was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (Kumar et al., 2004). The cell morphology and gram staining of the strain was examined after being cultured for 20 h by microscopy (magnification, ×1000). The utilization of some carbon sources by the strain was performed based on Bergey's Manual of Systematic Bacteriology (Kurane et al., 1986).

Analysis of antioxidative capacity

The antioxidant capacities were determined by four methods: 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH•) scavenging activity assay, total antioxidative capacity (T-AOC) assay, hydroxyl free radical –scavenging assay, superoxide anion radical-scavenging assay, Lipid peroxidation assay.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Free radical scavenging activity has a little modification base on 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), according to the method of Yen and Chen (1995). 0.5 ml of test sample was added to 3 ml of DPPH solution (0.2 mmol/L, in ethanol) and the mixture was shaken vigorously and incubated for 30 min at room temperature in darkness. The absorbance was measured at 517 nm using a spectrophotometer (SHIMADZU UV-1800). L-Ascorbic acid was used as a positive control. The free radical scavenging activity was then calculated as a percentage of inhibition according to the following equation:

$$\%\text{Inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where A = absorbance, A_{blank} = absorbance of the blank (DPPH solution plus ethanol water), and A_{sample} = absorbance of the test sample (DPPH solution plus the sample or L-ascorbic acid).

Total antioxidative capacity (T-AOC) assay

Total antioxidative capability (T-AOC) was measured with commercial assay kits from Nanjing Jiancheng Bioengineering Institute of China (No. 2002112). The T-AOC was measured by the method of ferric reducing-antioxidant power assay (Benzie and Strain, 1996) and detected at 520 nm with the spectrophotometer.

Hydroxyl free radical scavenging assay

Hydroxyl free radical scavenging activity was measured by Hydroxyl Free Radical (OH·) Detection Kit (Nanjing Jiancheng Bioengineering Institute, China). Hydroxyl free radical scavenging activity was measured by colorimetric determination of hydroxyl free radical from fenton reaction at 550 nm with a spectrophotometer. The protocol was carried out according to the manufacturer's instructions.

Superoxide anion radical scavenging assay

The superoxide anion radical scavenging ability was measured with a commercial assay kits from Nanjing Jiancheng Bioengineering Institute of China. The superoxide anion radical-scavenging was measured by the method of the active oxygen generation of xanthine-xanthine oxidase and detected at 550 nm with the spectrophotometer.

Lipid peroxidation assay

Lipid peroxidation (LPO) inhibitory activity was measured with a little modification according to the methods of Zhang et al. (1996). Egg lecithin (with equal volume phosphate buffer, 0.1 mol/L, pH 7.4) was stirred with a magnetic stirrer for 10 min, diluted with PBS (0.1 mol/L pH 7.4) in the ratio of 1:15 to obtain the egg lecithin suspension. The samples (0.5 ml) were added to 1 ml of egg lecithin suspension. Then 1 ml of PBS (0.1 mol/L pH 7.4) and 1 ml of 25 mmol/L $FeSO_4$ was added to induce lipid peroxidation. After 15 min of reaction at 37°C, the reaction was stopped by adding 1 ml of 2.5% (W/V) TCA. After centrifugation at 10,000 rpm for 20 min, 3 ml of supernatant was extracted and added to 2 ml of 0.8% TBA and then incubated in boiling water bath for 15 min. The absorbance of the supernatant was measured at 532 nm. The scavenging effect was calculated using the equation as described for DPPH.

$$\%\text{Inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where, A = absorbance.

Total phenolic content

Total phenolic content in the methanol extract was measured by the

Folin–Ciocalteau method with a little modification (Singleton and Rossi, 1965). A reaction mixture of 10 ml, contained 7.9 ml of double distilled water, 0.1 ml of sample and 0.5 ml of Folin–Ciocalteau reagent, followed by 1.5 ml of Na_2CO_3 (20%, W/V). After incubation for 30 min at 25°C, the absorbance was measured at 760 nm and the phenolic content was calculated with a gallic acid standard and expressed as gallic acid equivalents.

Selection of the best medium for antioxidant activity production

In order to select the best culture medium for optimal antioxidant activity production, several bacteria broth media were selected: nutrient broth (NB) with 1.5 g/L glucose, Luria–Bertani broth (LB) with 1.5 g/L glucose, TSB with 1.5 g/L glucose, and tryptic yeast (TY) with 1.5 g/L glucose, pH 7.0, autoclaving (121°C, 20 min). After inoculation, the strain was incubated at 30°C with shaking at 150 rpm for 3 days. Cell growth was monitored by optical density measurement at 600 nm, and antioxidant activity was tested by DPPH assay. All experiments were performed in triplicate (n = 3).

Kinetic production of antioxidant activity

Antioxidant activity was detected after incubating into NB medium with 1.5 g/L at 30°C during 120 h. Every 4 h during 24 h, and every 12 h from 24 to 120 h, the cell growth was monitored by optimal density and was measurement at 600nm, and DPPH assay of extracellular substance was also measured.

Effect of pH and heat treatment

Thermal stability of the antioxidant activity was evaluated by the incubation of XJ-25 at different temperatures for 30 min or after autoclaving at 121°C for 20 min. After cooling at room temperature, antioxidant activity was determined by DPPH assay. The effect of pH was determined by adjustment of XJ-25 pH from 2 to 12 with diluted HCl or NaOH. After incubation for 2 h at 30°C and neutralization to pH 7.0, the antioxidant activity was tested.

Solubility in organic solvents

XJ-25 was mixed with organic solvents, such as methanol, ethanol or acetone used at 4:1 ratio (v/v). The mixture stored at -20°C for 2 h followed by centrifugation at 12,000 rpm for 15 min at 4°C, both soluble and insoluble fractions were evaporated to dryness under speed-vacuum, then suspended in distilled water, and antioxidant activity was tested by DPPH scavenging assay.

Thin layer chromatography

Samples were spotted onto TLC silica gel 60 F254 (20×20 cm; layer thickness, 0.20 mm; Merck) plates, n-butanol-methanol-H_2O (25:15:10, v/v/v) was used as mobile phase, staining the developed plates after complete removal of the solvents. The Rf of the detected spots is defined as the ratio between the distance traveled by the compound divided by the distance traveled by the solvent.

Antioxidant activity staining method

To determinate the active antioxidant compound by XJ-25, a preliminary detection of the radical-scavenging activity was conducted (Sreenivasan et al., 2007). The developed TLC plate was sprayed with 0.1% w/v 1-diphenyl-2-picrylhydrazyl (DPPH) dissolved in methanol. The compound with antioxidant activity will appeared yellow spots against the purple-blue background. Dried plates were also treated with 0.3% (w/v) ninhydrin spray reagent dissolved in ethanol. Red spots were detected after incubation at 80°C for 10 min. Ninhydrin is the most widely used reagent for staining peptide with free N-terminal amino group (El-Thaher and Bailey, 1994).

Statistical analysis

All experiments were carried out in triplicates and repeated in three independent sets of experiments. Data are shown as means+standard deviation (SD). The Origin 8.0 version for windows computer programme was used for statistical analysis.

RESULTS AND DISCUSSION

Identification of the strain producing active antioxidant

XJ-25 was selected as a active antioxidant producing bacteria from different soils. A preliminary identification of XJ-25 by 16s rDNA analysis showed that the strain belonged to *Bacillus* genus. XJ- 25 was found to be a gram-positive, rod-shaped, aerobic bacterium and catalase positive. The colony of strain 25 in beef-protein medium is circular, smooth and white. The use of an API 50CHB kit with the APILAB Plus software indicated 90.01% identity with *B. simplex*. Partial sequence alignment of 16S rDNA confirmed biochemical data and identified XJ-25 strain as *B. simplex*. Recently, the antioxidant have aroused many researcher interest, with many studies focused on plant antioxidant extracts (Lee et al., 2010; Qingming et al., 2010), and Only a few reports have been conducted on antioxidant of microbial metabolic substances, especially in bacteria (Moktan et al., 2008; Tabbene et al., 2010). Basing on the molecular and biochemical analysis, XJ-25 strain was first screened and identified by us, as *B. simplex* which has never been reported to produce antioxidant active substances.

The screening of the best medium for active antioxidant production

Medium composition can affect the cell growth and production of many secreted products from micro-organism (Chen et al., 2008; Tabbene et al., 2009). Four different mediums NB, LB, TY, and TSB were tested for production of the antioxidant activity by *Bacillus simplex* XJ-25. The maximum antioxidant activity occurred in NB with 1.5 g/L glucose medium the maximum cell growth occurred in LB with 1.5g/L glucose. The DPPH inhibitory activity of XJ-25 in NB with 1.5 g/L glucose reached 92.86% (Figure 1). Culture of XJ-25 strain either in TY or TSB broth media resulted in prominent growth, but the DPPH inhibitory activity only achieved 20 and 23%

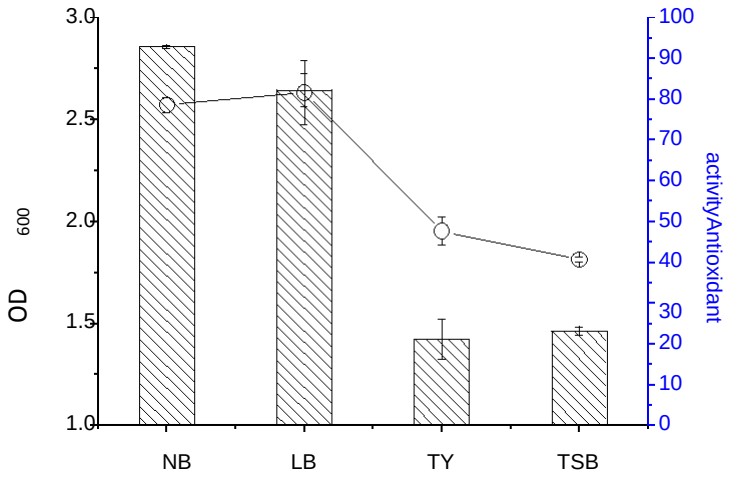

Figure 1. Effect of various growth media on cell growth and antioxidant activity production by Bacillus XJ-25 strain. NB, LB, TY, and TSB media were tested. Bacterial growth was evaluated by optical density measurement at 600 nm (open circles). Antioxidant activity measured by DPPH inhibitory activity (black bars).

respectively (Figure 1). This is clearly showed that optimal growth does not necessarily lead to a high level in antioxidant activity.

The antioxidant capacity of XJ-25

Nowadays, there are many methods used for evaluating the antioxidant activity. DPPH is a stable nitrogen-centered, lipophilic free radical which takes a relatively short time compared to other methods, therefore it is widely used in evaluating the antioxidant activities. The color of DPPH changing from violet to yellow upon reduction is demonstrated by the decrease of absorbance at 517 nm. Based on our result, DPPH scavenging activities of the extracellular secreted supernatant and the intracellular cell-free extracts of XJ-25 are shown in Table 1. The scavenging activity of the supernatant was 92.86%, and the intracellular cell-free extracts of XJ-25 is 8.00%. This is showed that DPPH scavenging activity was mainly obtained from extracellular metabolic com-pounds. This is quite different with the antioxidant activity of *lactobacilli,* which come mainly from intracellular cell-free extract (Jain et al., 2009).

T-AOC was measured by the method of ferric reducing-antioxidant power assay, total antioxidant capacity is a useful index of the combined action antioxidants in the body. The total antioxidative capacity of the extracellular secreted supernatant and the intracellular cell-free extracts of XJ-25 is 26.00 and 1.23 U/ml, respectively. The extracellular secret supernatant exhibited higher T-AOC than intracellular extracts.

The hydroxyl radical is one of the representative of the reactive oxygen species generated in the body. The highly reactive hydroxyl radical can cause oxidative damage to DNA, lipids and proteins (Spencer et al., 1994). The supernatant from XJ-25 of hydroxyl radical scavenging activity is 82.42%, but the percentage scavenging of intracellular cell-free extracts from XJ-25 is only 1.2% (Table 1). The supernatant of XJ-25 exhibited higher hydroxyl radical scavenging activity (82.42% diluted three times) than that of cell-free extracts. A very high hydroxyl ion scavenging ability suggests that, the extracellular secret supernatant has potentials of being used as alternative to synthetic antioxidants in arresting oxidative activity of hydroxyl ion.

Superoxide can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, they are very harmful to cellular components of biological systems (Zhao et al., 2006). Thus inhibiting superoxide capability could prevent superoxide accumulation. Superoxide anion scavenging activity of XJ-25 mainly showed on the supernatant (Table 1). The supernatant and the intracellular extracts of XJ-25 exhibited superoxide anion scavenging effect of 54.43 and 5%, respectively.

Lipid peroxidation can produce many aldehydes products and cause cell membrane disruption and cell damage (Barrera et al., 2008). This process forms the peroxy radicals (LOO·) initiated by hydroxyl- and superoxide-radicals. Thus, antioxidants capable of scavenging peroxy radicals could prevent lipid peroxidation. Lipid peroxidation is another factor to indicate the antioxidant activity. In our study, the supernatant demonstrated a higher antioxidant activity (41.81%) than the intracellular cell-free extracts of XJ-25 (Table 1).

Based on the result of DPPH assay, T-AOC assay, hydroxyl radical assay, Superoxide anion scavenging activity assay and lipid peroxidation, the main functional compounds as secondary metabolites was in the

Table 1. The antioxidant activity of XJ-25.

Sample	DPPH radical scavenging activity (%)	Total antioxidative capacity (U/ml)	Hydroxyl free radical scavenging activity (%)	Superoxide anion radical-scavenging activity (%)	Lipid peroxidation assay (%)
25 (extracellular secreted)	92.86±1.09	26.00±0.97	82.42[a]±2.12	54.43±0.23	41.81±0.21
25 (intracellular cell-free extracts)	8.00±0.02	1.23±0.02	1.20±0.11	5.00±0.12	0
VC	90.61±1.78	25.82±1.23	48.79±0.97	49.92±0.12	34.51±0.09

a, 25 sample diluted with water in the ratio of 1:3.

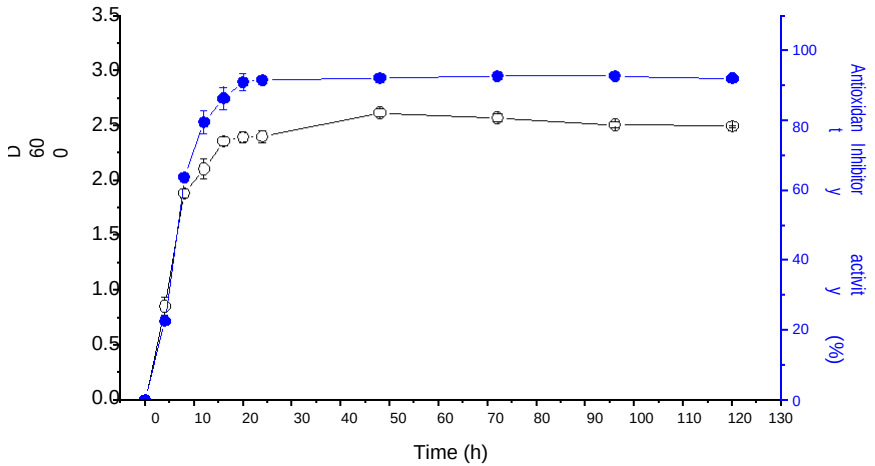

Figure 2. Kinetic study of antioxidant activity production by XJ-25 strain. Cell growth was measured by optical density at 600 nm. The antioxidant activity was expressed by DPPH inhibitory activity. Closed circles DPPH inhibitory activity; open circles OD600.

supernatant. The result is different with *Bifidobacterium longum* ATCC 15708 in which the main active compounds lay in the intact cells and intracellular cell-free extracts (Lin and Chang, 2000).

Based on our result, total phenolic content in the supernatant of XJ-25 was 18.71±0.10 ug gallic acid equivalents/ml. Antioxidant activity is often associated with phenolic compounds present in the plant extract (Qingming et al., 2010). Polyphenols are mainly from the methanol extracts in many leaves and stems of plants with antioxidant activity (Adedapo et al., 2009), which can absorb and neutralise free radicals, quench singlet and triplet oxygen, or decompose peroxides.

Kinetic assay of the antioxidant activity production

The kinetic assay of the antioxidant activity during 120 h of the XJ-25 in NB medium with 1.5 g/L glucose was investigated; aliquots of culture medium were sampled at various time intervals. The cell growth reached the stationary phase 16 h after inoculation. The DPPH inhibitory activity reached an optimal level of 90.01%, 20 h after inoculation (Figure 2). Synthesis of antioxidant compounds by XJ-25 reaches its maximum level during the stationary phase. These results suggested that the production of active compounds by XJ-25 is highly dependent on the

Table 2. Effect of temperature, pH and organic solvents treatment on the antioxidant activity from XJ-25.

Treatments	DPPH radical-scavenging activity (%)	Treatments	DPPH radical-scavenging activity (%)	Treatments	DPPH radical-scavenging activity (%)
Control	**84.81**	**pH**		**Organic solvents**	
Temperature		2	82.48	Methanol	87.07
40°C for 30 min	82.71	3	81.85	Ethanol	86.23
50°C for 30 min	86.92	4	83.96	Acetone	83.23
60°C for 30 min	86.99	5	82.55		
70°C for 30 min	87.07	6	77.18		
80°C for 30 min	86.76	7	81.07		
90°C for 30 min	86.60	8	74.45		
100°C for 30 min	85.51	9	71.88		
Antoclaveing 121°C for 20 min	87.62	10	69.39		
		11	84.35		
		12	87.07		

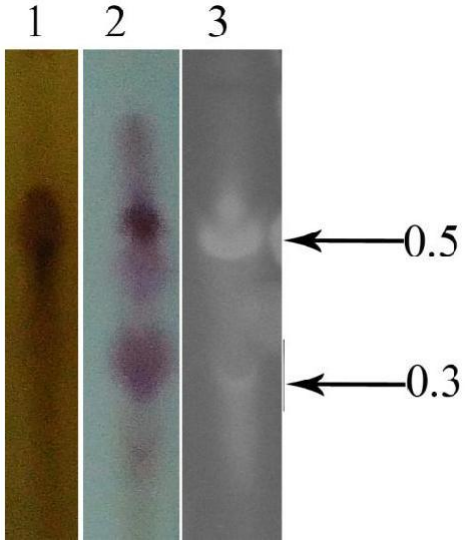

Figure 3. TLC-antioxidant activity assay produced by XJ-25. Iodine fuming (lane 1), ninhydrin (lane 2), DPPH (lane 3). Arrows indicated Rf values of bioactive compounds.

the growth phase period. This also showed that XJ-25 strain was able to secrete a bioactive compound as soon as the early growth phase. Similar bioactive compounds production pattern has also been reported in other *Bacillus* species (Naclerio et al., 1993).

Physico-chemical properties of the antioxidant activity

To test the stability of the active antioxidant compounds, various pH, temperature and different organic solvents were used in the experiment. Our results indicated that, the antioxidant activity under different treatments of XJ-25 have no significant difference compared to the control B (Table 2). The heat-stable property was also observed in other antioxidant compound (Aristoy et al., 2004). The stability of this antioxidant activity over heating and a wide range of pH treatment might be useful in several industrial applications.

To further characterize the antioxidant activity, the extracellular secreted supernatant of XJ-25 was subjected to TLC analysis, and biological properties of the separated bioactive compounds were investigated. At least two bioactive spots were observed in TCL chromatography. One spot with Rf value of 0.55 shows a higher antioxidant activity than the other spot with Rf value of 0.37. This indicated that the active compound with Rf value of 0.55 counts for the main antioxidant activity. The separate active spots were also stained with the ninhydrin and iodine fuming. Both spots with Rf value of 0.55 and 0.37 were ninhydrin positive (Figure 3). Since both spots were ninhydrin positive, a free amino group might exist in these active compounds.

Conclusion

B. simplex XJ-25 was first isolated by us from

sand biological soil crusts, produced secondary antioxidant activity metabolites. The active compounds display a significant stability towards temperature, pH and organic solvents. This might be significantly useful in the development of new antioxidant activity compound. Further research should be carried on to purify the active compound in order to expatiate on the exact chemical structures.

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