Tyrosinase inhibitors and insecticidal materials produced by *Burkholderia cepacia* using squid pen as the sole carbon and nitrogen source

Chia-Hao Hsu[a], Anh Dzung Nguyen[b], Yung-Wei Chen[a], and San-Lang Wang[a,c*]

[a]*Department of Chemistry, Tamkang University, 251 New Taipei, Taiwan*
[b]*Biotechnology Centre, Tay Nguyen University, 567 Buon Ma Thuot, Vietnam*
[c]*Life Science Development Center, Tamkang University, 251 New Taipei, Taiwan*

*Corresponding author. Present address: 151 Ying-Chuan Road, Tamsui, New Taipei 25137, Taiwan. Tel.: +886 2 2621 5656; fax: +886 2 2620 9924.

E-mail: address: sabulo@mail.tku.edu.tw (S.-L. Wang).

ABSTRACT

Reports of tyrosinase inhibitors from microorganisms are rare. A tyrosinase inhibitor- and insecticidal materials-producing bacterium, strain TKU026, was isolated from Taiwanese soil and identified as *Burkholderia cepacia*. Among the tested chitin-containing materials, squid pen best enhanced the production of tyrosinase inhibitors and insecticidal materials. The tyrosinase inhibitory activity (5000 U/mL) and insecticidal activity (81%) against *Drosophila* larvae was maximised after cultivation on 1% squid-pen containing medium for three days. The tyrosinase inhibitory activity persisted even when the culture was treated with acidic or alkaline conditions of pH 3 or 11. The activities of both tyrosinase inhibitors and insecticide remained at 100%, even after treatment at 100℃ for 30 min. The culture supernatant after three days of cultivation also showed antifungal activity against *Aspergillus fumigatus* and *Fusarium oxysporum* with maximal activities of 100% and 80%, respectively, but no antibacterial activity against *Escherichia coli* was observed. The tyrosinase inhibitors were assumed to be polyphenolic compounds according to the results of chromatography.

Keywords:
Tyrosinase inhibitor, squid pen, insecticide, *Burkholderia cepacia*

1. Introduction

Tyrosinase (EC 1.14.18.1) is a multifunctional copper-containing enzyme that is widely distributed in fungi, plants, and animals and is responsible for melanin biosynthesis [1] as well as the browning reaction in fruits, vegetables or seafood [2] and human skin pigmentation abnormalities [3]. In addition, tyrosinase is also an important enzyme in the process of insect moulting [4]. Melanogenesis can be controlled by tyrosinase inhibition or by blocking the maturation processes of tyrosinase and its related proteins [5]. Most tyrosinase inhibitors, such as diphenol, 4-methyl-catechol, azelaic acid [6], hydroquinone, arbutin [7], dihydroasparagusic acid [8], 4-*n*-butylresorcinol [9], and oxyresveratrol [10], are phenolic compounds. Hyperpigmentary disorders, such as melisma or actinic and senile lentigine, are a major cosmetic concern [9]. On food, the products of tyrosinase activity can also lead to deleterious effects, such as browning and the postharvest disease of fruits [11], and the products of tyrosinase activity associated with browning reactions in vegetables and black spotting of shrimp and lobsters [2] can be reduced using a tyrosinase inhibitor.

Most tyrosinase inhibitors are also somewhat toxic to humans; therefore, finding less toxic compounds has been the primary objective in recent studies. Natural sources have been widely investigated as a source of tyrosinase inhibitors, including plants, animals, microorganisms, and other marine organisms, and these compounds are largely free of harmful adverse effects [3]. Tyrosinase inhibitors produced by microbial fermentation have rarely been reported, such as those from the fungi of *Trichoderma* sp. H1-7 [12], *Aspergillus oryzae* BCRC 32288 [3], *Myrothecium* sp. MFA58 [13], fungus YL185 [14] and the bacterium of *Streptomyces roseolilacinus* NBRC 12815 [15].

Squid pen, a chitin-containing fishery processing byproduct, contains chitin and protein and is valuable to the production of bioactive materials when reutilised by microbial conversion [16, 17]. Squid pen has been used as the sole carbon/nitrogen source to isolate tyrosinase-producing bacteria from Taiwanese soil in order to recycle chitinous materials. In this study, we reported the isolation of a tyrosinase inhibitor-producing strain TKU026 which was identified as *Burkholderia cepacia*. In addition to tyrosinase inhibitory activity, insecticidal activity against *Drosophila* larvae and antifungal activity were also found in the culture supernatant of squid pen powder-containing medium.

B. cepacia can reportedly improve seed germination and seedling growth in maize (*Zea mays*) and rice (*Oryza sativa*) [18] and can produce some antibacterial agents to inhibit bacteria, fungi and some plant pathogens, such as pyrrolnitrins, altericidins, and cephamycins, which can effectively control the bacterial wilt of tobacco (tobacco wilt) and other plant diseases [19]. However, the tyrosinase inhibitors and insecticidal materials produced by *B. cepacia* have not been reported. Therefore, the aim of this study was to investigate the culture conditions for the production of tyrosinases and insecticidal materials, as well as identify the properties of the tyrosinase inhibitors.

2. Experimental

2.1 Materials

Shrimp heads, Katsuobushi from mackerel, Katsuobushi from bonito, crab chitin, and squid pen were all purchased from Shin-Ma Frozen Food Company (I-Lan, Taiwan). The dried materials were milled to a powder for use as the carbon/nitrogen source for the production of tyrosinase inhibitors. L-DOPA, mushroom tyrosinase, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma.

2.2 Isolation and screening of tyrosinase inhibitor-producing strains

Soil samples were diluted with sterile water, and medium containing 1% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4.7H_2O$ and 1.5% agar was inoculated with the suspensions. After cultivation at 37℃ for two to three days, the observed colonies were screened and used to inoculate liquid medium containing 1% SPP, 0.1% K_2HPO_4, and 0.05% $MgSO_4.7H_2O$. After cultivation at 37℃ on a rotary shaker (150 rpm) for 2–3 days, the culture was harvested by centrifugation at 10000 *g* for 10 min, and the supernatant was used to assay the tyrosinase inhibitory activity.

2.3 Identification of the strain TKU026

According to the result of a 16S rDNA partial base sequence (Figure 1) and the API identification system, TKU026 is closest to *Burkholderia cepacia*, the similarity of the 16S rDNA partial base sequence was more than 99.9%

2.4 Measurement of tyrosinase inhibitory activity

The reaction media (0.9 mL) for the activity assay contained 590 μl of 4.5 mM $_L$-DOPA in 25 mM sodium phosphate buffer (pH 7) and 300 μl of supernatant,

which was then pre-incubated at 25℃ for 3 min. Subsequently, an enzyme solution (3200 U/mL of tyrosinase, 10 μl) was added. After 2 min of incubation at 25℃, the absorbance of the mixture was determined at 475 nm using a spectrophotometer [20].

The percentage of inhibition was defined as ((A /B)-1)×100%，where B is the increased optical density without the inhibitor after 2 min of reaction and A is the increased optical density with the inhibitor after 2 min.

2.5 Preparation of concentrated culture supernatant

After cultivation in liquid medium containing 1% SPP, 0.1% K_2HPO_4, and 0.05% $MgSO_4.7H_2O$ (pH 7) at 37℃ on a rotary shaker (150 rpm) for 3 days, the culture supernatant (5600 mL) obtained by centrifugation at 10000 *g* for 10 min was concentrated by ammonium sulphate precipitation (600 g/L). The resultant precipitate was dissolved in an appropriate amount of water and then dialyzed against 25 mM sodium phosphate buffer (pH 7) at 4℃ overnight. The dialyzed solution (280 mL) was then used as a 20-fold-concentrated culture supernatant.

2.5. Antifungal test

Two fungi stocked in our laboratory, *Aspergillus fumigatus* (AF) and *Fusarium oxysporum* (FO), were used for the antifungal test. After cultivation on potato dextrose agar (PDA) at 30℃ for one day, the concentrated culture supernatants were inoculated with the fungi for antifungal testing. Deionised water was used as a control.

2.6 Determination the content of total polyphenol

The resulting solution (100 μL) was added to 2 mL of 2% Na_2CO_3. After 2 min, 50% Folin-Ciocalteu reagent (100 μL) was added to the mixture, which was then incubated for 30 min. The absorbance was measured at 750 nm using a spectrophotometer [21]. The results are expressed as milligrams per gram of gallic acid equivalents (GAEs).

2.7 Partial purification of the tyrosinase inhibitor

Ammonium sulphate (608 g/L) was added to the culture supernatant (660 mL). The resulting mixture was stored at 4℃ overnight, and the resultant precipitate was collected by centrifugation at 4℃ for 20 min at 12,000 *g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7) and dialysed against the buffer. The resulting dialysate (20 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). The column was washed with the same buffer and eluted

with a linear gradient of 0-1 M NaCl in the same buffer. The fractions containing the tyrosinase inhibitory activity peaks were independently pooled and concentrated using ammonium sulphate precipitation. The pooled inhibitor solution fractions were purified further. The obtained inhibitor solution (the tyrosinase fractions from the DEAE-Sepharose CL-6B column) was then chromatographed on a Sephacryl S-100 column (2.5 cm × 120 cm) that had been equilibrated with 50 mM sodium phosphate buffer (pH 7). The inhibitor was eluted using the same buffer. The fractions containing inhibitory activity were pooled and used as a purified preparation.

3. Results

3.1 Tyrosinase inhibitors production

To investigate the culture conditions that enhance tyrosinase inhibitors production, bacteria were grown in a medium containing 1% (w/v) of a carbon/nitrogen (C/N) source, 0.1% K_2HPO_4, and 0.05% $MgSO_4.7H_2O$ at 30℃ for 1–5 days of fermentation in Erlenmeyer flasks on a rotary shaker at 150 rpm. The C/N sources used were squid pen powder (SPP), shrimp head powder (SHP), katsuobushi from mackerel (KM), katsuobushi from bonito (KB), crab chitin powder (CCP), and nutrient broth (NB). After cultivation, the culture was harvested by centrifugation, and the supernatant was used to assay the tyrosinase inhibitory activity. As shown in Table 1, SPP was the best C/N source for tyrosinase inhibitor production (5000 U/mL); the productivity was higher when using SPP than when using commercial medium NB (2890 U/mL) or other tested C/N sources. Among these other sources, the cell growth and tyrosinase inhibitory activity of CCP (lower than 10 U/mL) was the worst. This result may be related to the lower protein content that could not provide sufficient C/N sources for cell growth.

3.2 Insecticidal materials production

Tyrosinase has been reported as an important enzyme in the process of insect moulting [4]. To investigate whether the TKU026 produced insecticidal materials, the insecticidal activity of the culture supernatant against *Drosophila* larvae was also studied. Although a higher productivity of tyrosinase inhibitory activity (5000 U/mL) was found in 25 mL of medium, 100 mL of medium (3350 U/mL) was used in the following experiments based on production cost. As shown in Figure 2, the insecticidal activity (80%) and cell growth were maximised on the second day, and the tyrosinase inhibitory activity (3350 U/mL) was maximised on the third day.

3.3 Antifungal test

B. cepacia can reportedly improve seed germination and seedling growth in maize (*Zea mays*) and rice (*Oryza sativa*) [18] and produce antibacterial agents to inhibit bacteria, fungi and some plant pathogens.

The 20-fold-concentrated culture supernatant prepared via Method 2.5 was diluted with an appropriate amount of sterilised distilled water to prepare different concentrated concentrations of culture supernatant of *B. cepacia* TKU026 for the antifungal test. These antifungal samples were mixed with the same volume of sterilised potato dextrose agar (PDA) and poured into Petri dishes. After inoculum the testing the fungi, i.e., *Aspergillus fumigatus* (AF) and *Fusarium oxysporum* (FO), and incubated them at 30℃ for three days, the inhibitory effect was observed and is shown in Table 2. The culture supernatants inhibited both of the tested fungi, with maximal inhibitions of 100% and 80% of AF and FO, respectively.

3.4 Some physical properties of the tyrosinase inhibitors

To examine the thermal stability of TKU026 tyrosinase inhibitors, the culture supernatant was incubated for 30 min at various temperatures in 50 mM phosphate buffer (pH 7), and the residual activity was then measured. TKU026 tyrosinase inhibitors were remarkably thermostable and retained 100% of their initial activity from 25 to 100℃ (data not shown). In addition, the TKU026 tyrosinase inhibitors pH stability profiles were determined by a residual activity measurement at pH 7 after incubation at various pH values at 37℃ for 30 min. The TKU026 tyrosinase inhibitors were relatively stable at pH 3-11 and retained 100% of the initial activity (data not shown).

3.5 Total polyphenolic content

As shown in Figure 3, similar to the results of the tyrosinase inhibitory activity, the total polyphenol content increased with the cultivation time. The highest total polyphenol content (1012 mg/mL GAE) and tyrosinase inhibitory activity (3333 U/mL) were both found on the third day. According to the results, the TKU026 tyrosinase inhibitor was suspected to be a phenol-containing non-proteinaceous compound. Therefore, the TKU026 tyrosinase inhibitor was then partially purified from the culture supernatant.

3.6 Partial purification of the tyrosinase inhibitor

As shown in the results of the DEAE-Sepharose column chromatography (Figure 4), the TKU026 tyrosinase inhibitor was not related to protein-containing materials. Sephacryl S-100 gel chromatography was performed to further purify the TKU026 tyrosinase inhibitor. As shown in Figure 5, the tyrosinase inhibitory activity coincided with the total phenol and DPPH scavenging ability peaks. According to the above results, we assumed that the TKU026 tyrosinase inhibitor may be a phenol-containing compound.

DISCUSSION

In this study, we found that squid pen was a potential carbon/nitrogen source for tyrosinase inhibitor production by *Burkholderia cepacia* TKU026, a bacterium isolated from Taiwanese soil. As summarised in Table 3, *B. cepacia* TKU026 resulted in the highest tyrosinase inhibitory activity with the cheapest culture medium compared with the other reports of tyrosinase inhibitor-producing strains [3, 12, 13, 15, 24]. In addition to tyrosinase inhibitors, the culture supernatant of *B. cepacia* TKU026 also showed insecticidal activity against *Drosophila* larvae and antifungal activity against *A. fumigatus* and *F. oxysporum*. To the best of our knowledge, tyrosinase inhibitors and/or insecticidal materials produced by *B. cepacia* have not been reported.

Similar to most of the other tyrosinase inhibitors [6, 7, 22, 23], TKU026 tyrosinase inhibitors were also suspected to be phenol-containing compounds according to the results of a partial purification by column chromatography. Commercial tyrosinase inhibitors, such as kojic acid, hydroquinone, resveratrol, and arbutin, are suspected to be toxic. The tyrosinase inhibitors with insecticidal and antifungal activities partially purified from the culture supernatant of *B. cepacia* TKU026 could potentially be used in the cosmetic, agricultural, and food industries.

ACKNOWLEDGEMENTS

This work was supported in part by a grant ~~o~~from the National Science Council, Taiwan (NSC 102-2313-B-032-001-MY3, NSC 102-2621-M-032-005, and NSC 102-2811-B-032-001).

REFERENCES

1. Y.J. Kim, H. Uyama, Cell. Mol. Life Sci. **62**, 1707–1723 (2005)
2. E. Neeley, G. Fritch, A. Fuller, J. Wolfe, J. Wright, W. Flurkey, Int. J. Mol. Sci. **10**, 3811–3823 (2009)
3. T.S. Chang, H.Y. Ding, S.S.K. Tai, C.Y. Wu, Food Chem. **105**, 1430–1438 (2007)
4. M. Miyazawa, and N. Tamura, Biol. Pharm. Bull. **30**, 595–597 (2007)
5. W.R. Hyung, S.H. Jeong, M.J. Curtis-Long, S. Jung, J.W. Lee, H.S. Woo, J.K. Cho, K.H. Park, J. Agric. Food Chem. **60**, 8372–8378 (2012)
6. K.U. Schallreuter, J.W. Wood, Arch. Derm. Res. **282**, 168–171 (1990)
7. K. Maeda, M. Fukuda, J. Soc. Cosmetic .Chem. **42**,361–368 (1991)
8. A. Venditti, M. Mandrone, A.M. Serrilli, A. Bianco, C. Iannello, F. Poli, F. Antognoni, J. Agric. Food Chem. **61**, 6848–6855 (2013)
9. L. Kolbe, T. Mann, W. Gerwat, J. Batzer, S. Ahlheit, C. Scherner, H. Wenck, F. Stäb, JEADV. **27**,19–23 (2013)
10. C. Liang, J.H. Lim, S.H. Kim, D.S. Kim, Food chem. **134**, 1146–1148 (2012)
11. Z.Z. Pan, Y.J. Zhu, X.J. Yu, Qi.F. Lin, R.F. Xiao, J.Y. Tang, Q.X. Chen, Bo. Liu, J. Agric. Food Chem. **60**, 10784–10788 (2012)
12. C. Imada, Y. Sugimoto, T. Makimura, T. Kobayashi, N. Hamada, E. Watanabe, Fisheries Sci. **67**, 1151–1156 (2001)
13. X. Li, M.K. Kim, S.K. Kim, S.J. Kang, U. Lee, H.D. Choi, B.W. Son, Chem. Pharm. Bull. **53**, 453–455 (2005)
14. K. Shimizu, X. Geng, M. Hashiguchi, H. Suhara, S. Fukunaga, S. Yasutake, R. Kondo, M. Tsutui, I. Sato, J. Wood Sci. **49**, 349–354 (2003)
15. T. Nakashima, K. Anzai, N. Kuwahara, H. Komaki, S. Miyadoh, S. Harayama, M.D.B. Tianero, J. Tanaka, A. Kanamoto, K. Ando, Biol. Pharm. Bull. **32**, 832–836 (2009)
16. S.L. Wang, T.W. Liang, Y.H. Yen, Carbohydr. Polym. **84**, 732–742 (2011)
17. S.L. Wang, Biocatalysis Agric. Biotechnol. **1**, 177–180 (2012)
18. A.H. Rodrı´guez, M.H. Pe´rez, B. Diallo, M.E. Jaziri, O.M. Vandeputte, Plant Growth Regul. **60**, 191–197 (2009)
19. J.R.W. Govan, J.E. Hughes, P. Vandamme, JMM. **45**, 395–407 (1996)
20. C.K. Hsu, C.T. Chang, H.Y. Lu, Y.C. Chung, Food Chem. **105**,1099–1105 (2007)
21. G.C. Yen, C.L. Hsieh, J. Agric. Food Chem. **46**, 3952–3957 (1998)
22. T. Sabudak, O. Demirkiran, M. Ozturk, G. Topcu, Phytochem. **96**, 305–311 (2013)
23. Y. Kang, J.U. Choi, E.A. Lee, H.R. Park, Food Sci. Biotechnol. **22**, 1449–1452

(2013)
24. B. Xu, Y. Yu, P. Wan, C. Wan, S. Cao. Res. Chem. Intermed. DOI10.1007/s11164-013-1154-8

FIGURE LEGENDS

Figure 1. The partial/16S rDNA sequence of *B. cepacia* TKU026

Figure 2. Time course of tyrosinase inhibitory activity and insecticidal activity produced by *B. cepacia* TKU026

Figure 3. Time course of tyrosinase inhibitory activity and total polyphenolic compounds produced by *B. cepacia* TKU026

Figure 4. DEAE-Sepharose column chromatography of *B. cepacia* TKU026 tyrosinase inhibitors

Figure 5 Sephacryl S-100 gel chromatography of *B. cepacia* TKU026 tyrosinase inhibitors

CGGGGCTGCTTACCATGCAGTCGAACGGCAGCACGGGTGCTTG
CACCTGGTGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACATGTCCT
GTAGTGGGGGATAGCCCGGCGAAAGCCGGATTAATACCGCATACGATCTAC
GGATGAAAGCGGGGGACCTTCGGGCCTCGCGCTATAGGGTTGGCCGATGG
CTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCAGTAGC
TGGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGAAAGCCTGA
TCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTT
GTCCGGAAAGAAATCCTTGGCTCTAATACAGTCGGGGGATGACGGTACCGG
AAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGTTTG
CTAAGACCGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTGGTGAC
TGGCAGGCTAGAGTATGGCAGAGGGGGGTAGAATTCCACGTGTAGCAGTG
AAATGCGTAGAGATGTGGAGGAATACCGATGGCGAAGGCAGCCCCCTGGG
CCAATACTGACGCTCATGCACGAAAGCGTGTGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGTTGTTGGGGATTCATTT
CCTTAGTAACGTAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCG
CAAGATTAAAACTCAAAGGAATTGACGGGACCCGCACAAGCGGTGGATGA
TGTGGATTAATTCGATGCAACGCGAAAACCTTACCTACCCTTGACATGGTCG
GAATCCTGCTGAGAGGGGGGAGTGCTCGAAAGAGAACCGGCGCAC

Figure 1

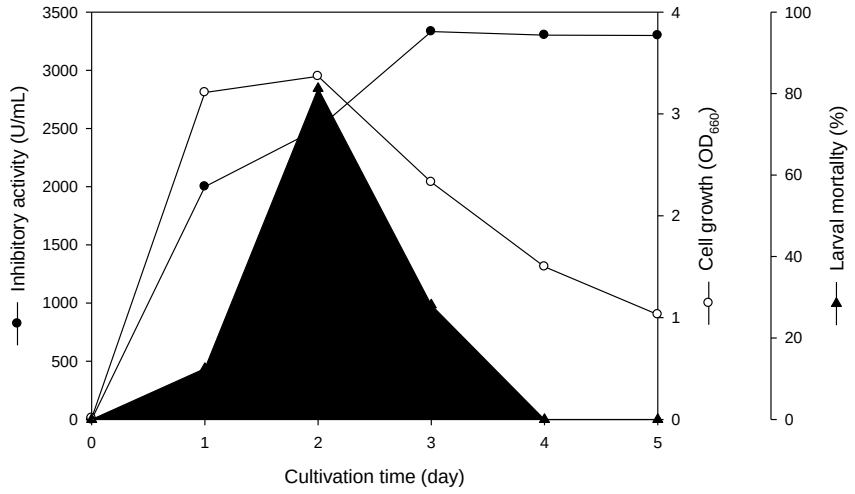
Inhibitory activity (U/mL)
Cell growth (OD660)
Larval mortallty (%)
Cultivation time (day)
0
500
1000
1500
2000
2500
3000
3500
1
2
3
4
20
40
60
80
100
5

Figure 2

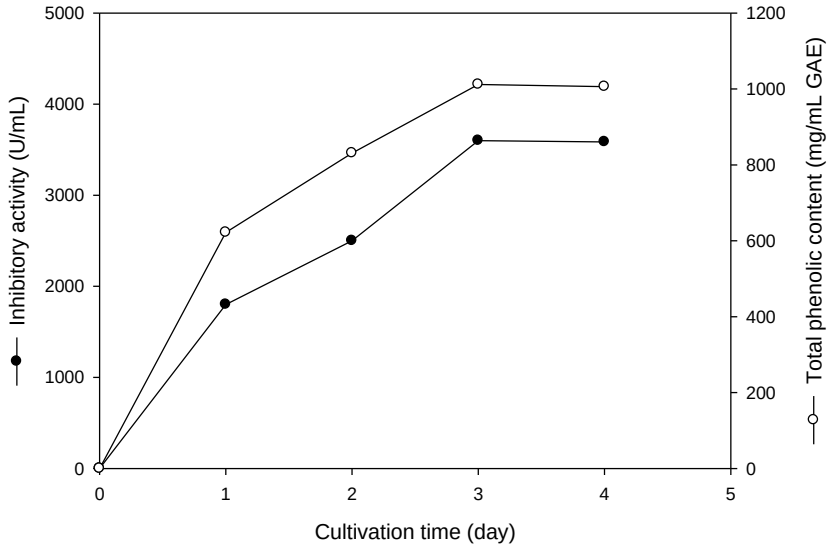
Inhibitory activity (U/mL)
Total phenolic content (mg/mL GAE)
Cultivation time (day)
0
1000
2000
3000
4000
5000
0
200
400
600
800
1000
1200
0
1
2
3
4
5

Figure 3

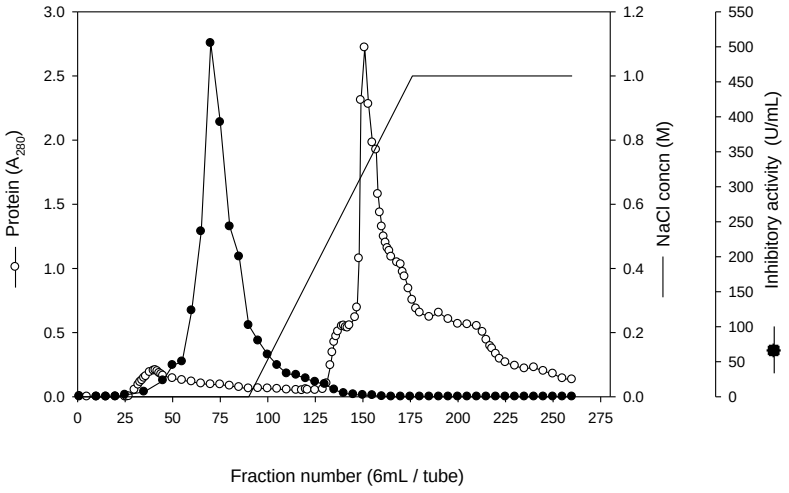
Protein (A_{280})
NaCl concn (M)
Inhibitory activity (U/mL)
Fraction number (6mL / tube)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
550
500
450
400
350
300
250
200
150
100
50
0
0
25
50
75
100
125
150
175
200
225
250
275

Figure 4

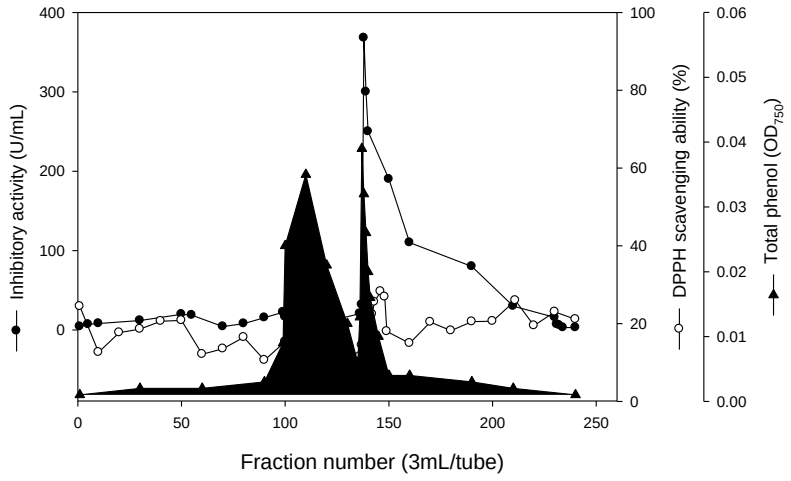
Inhibitory activity (U/mL)
DPPH scavenging ability (%)
Total phenol (OD750)
Fraction number (3mL/tube)
400
300
200
100
0
100
80
60
40
20
0
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
50
100
150
200
250

Figure 5

Table 1. Optimum culture conditions for TKU026 tyrosinase inhibitors production

C/N	Concentration (%)	Culture volume (mL)	Cultivation time (day)	Inhibitory activity (U/mL)
NB		100	3	2890
SPP	1	25	3	5000
SHP	1	100	3	3030
CCP	1	100	3	<10
KM	1	100	3	3060
KB	1	100	3	2390

Table 2. Antifungal effect of TKU026 culture supernatant on *A. fumigatus* and *F. oxysporum*.

Concentration (%)	Inhibition (%) on	
	A. fumigatus	*F. oxysporum*
0	0	0
0.5	60	50
1	75	60
1.5	90	75
2	100	80

Table 3 Comparison of C/N source and productivity of tyrosinase inhibitory activity by different microorganisms

Strains	C/N	Inhibitory activity		References
		Productivity (U/mL)	IC50 (μ m)	
Burkholderia cepacia TKU 026	1% SSP	5000	-	This study
Aspergillus oryzae BCRC 32288	rice, soybean, or sorgerm	-	212	3
Trichoderma sp. H1-7	2% soluble starch 1% glucose 0.5% Bacto-yeast extract 0.5% Tripticase peptone	1000-2500	-	12
Myrothecium sp. MFA58	Seawater-based medium	-	6.6	13
Fungus YL185	0.5% peptone 0.5% malt extract 0.2% yeast extract 1.5% glucose	-	-	14
Streptomyces roseolilacinus NBRC12815	2.5% starch 1.5% soy bean meal 0.2% dry yeast	-	1086	15