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Evaluation of Putative Inducers and Inhibitors toward Tyrosinase from two *Trichoderma* species

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Abstract

The present investigation deals with the purification of extracellular tyrosinase produced by *Trichoderma reesei* and *Trichoderma harzianum* and the characterization of putative inducers and inhibitors on the enzyme activity. Tyrosinase enzyme from *T. reesei* and *T. harzianum* was purified to homogeneity using ammonium sulphate precipitation (85%) followed by DEAE-cellulose, and phenyl Sepharose. SDS-PAGE exhibited a single band of 45 and 65 kDa for the purified tyrosinase from *T. reesei* and *T. harzianum*, respectively. The influence of guaiacol, catechol, vanillin, caffeic acid, syringaldazine and *p*-coumaric acid on purified enzyme was tested. Tyrosinase produced by the two Trichoderma species displayed a dose-dependent response toward all tested inducers. The effect of kojic acid, cinnamic acid, sodium azide, benzaldehyde and potassium cyanide was assayed at pH 5.0 in presence of monophenolic substrate (*p*-coumaric acid) and diphenolic substrate (caffeic acid). KCN and kojic acid were the powerful inhibitors on the tyrosinase-monophenolase and diphenolase activities from the investigated *Trichoderma*, respectively. The rate of tyrosinase activity showed a pseudo-first-order reaction kinetics and was proportional to the inhibitor concentration. The results reveal the possibility of using the inducers of tyrosinase to increase its activity.

Keywords: *Trichoderma*, Tyrosinase, SDS-PAGE, Inducers, Inhibitors.

1. Introduction

Tyrosinases (EC 1.14.18.1) are binuclear metallo-enzymes containing copper. Tyrosinases catalyze two different oxidation reactions through performing two functionality enzyme activities. Firstly, monophenols oxidize to *o*-diphenols (monophenolase activity). Secondly, *p*-substituted *o*-diphenols are oxidized to the *o*-quinones (diphenolase activity). In both oxidation reactions, oxygen acts as a terminal electron acceptor with the concomitant reduction to water and the formation of free radicals (El-Shora and Metwally, 2008; Fairhead and Thony-Meyer, 2012; Pretzler *et al*., 2017; Wang *et al*., 2018).

Tyrosinases as common enzymes in nature are widely distributed among plants, animals and microbes (Chang, 2009; Nawaz *et al*., 2017). It can perform various functions for plants, like production of lignin, tannins and flavonoids compounds as well as control of redox potential in plant respiration process (Marusek *et al*., 2006; Selinheimo *et al*., 2007a) and for humans, like melanin production (Zaidi *et al*., 2014; Wang *et al*., 2018). The majority of fungal tyrosinases are intracellular enzymes (Kong *et al*. 2000; Nakamura *et al*. 2000; Nawaz *et al*., 2017). However, the extracellular fungal tyrosinases have also been characterized (Montiel *et al*., 2004). The best characterized intracellular tyrosinase is derived from *A. bisporus* and *Neurospora crassa* (Ismaya *et al*., 2011), while the best characterized extracellular enzyme is derived from *Trichoderma reesei* (Selinheimo *et al*., 2006).

Several compounds may elicit a positive effect on tyrosinase production. These compounds are called inducers which include phenolic, aromatic compounds and metal ions. Substrates-specificity range varies from one type of tyrosinase to another. The variation of tyrosinase physiological functions may be the result of substrate specificity of tyrosinase. Tyrosinases are capable of oxidizing an extensive range of phenolic compounds. Tyrosinases can also oxidize peptide- and protein-containing a tyrosyl residues (Gasowska *et al*. 2004; Halaouli *et al*. 2005; Selinheimo *et al*. 2007a,b).

Endogenous tyrosinase oxidizes the phenolic compounds found in fruit and vegetables tissues. Tyrosinase-catalyzed browning reaction cause harmful changes in food appearance and properties (Lertsiri *et al*., 2003; Nawaz *et al*., 2017). Browning reactions cause a subsequent shortening of a product's shelf life. Anti-browning agents offer protection for the plant parts against toxic phenolic compounds. They may be either enzyme inhibitors or reducing agents. The elementary step in oxidation reaction of a phenolic compounds by tyrosinase can be suppressed or prevented by various enzyme inhibitors (Seo *et al*. 2003; Kim and Uyama 2005; Chang, 2009).

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Since there is little information about tyrosinase, particularly from *Trichoderma harzianum*, the present work focused on the purification and evaluation of the substrate-specificity of tyrosinase obtained from *Trichoderma reesei* and *Trichoderma harzianum*. In addition, the effects of various putative inhibitors on *Trichoderma* tyrosinase were evaluated.

2. Materials and Methods

2.1. Chemicals

All products were of analytical grade and obtained from different companies such as Sigma-Aldrich, Fluka and Merk.

2.2. Microorganism and culture maintenance

The investigated two fungi, *Trichoderma reesei* and *Trichoderma harzianum*, were provided from the Culture Collection and Identification Unit at the Regional Center for Mycology and Biotechnology, Al- Azhar University, Egypt. Fungal strains were grown on malt extract broth supplemented with phenol (pH 7.0). Cultures were inoculated in 100 mL medium and incubated for 3 days at 27 °C with shaking at 180 rpm. For extended periods of storage, fungi were sub-cultured periodically on malt extract agar plates.

2.3. Enzyme preparation

Fungal mycelium (30g) was collected through filtration using Whatman filter paper No. 1 (Whatman, Piscataway, NJ, USA). Mycelium debris was removed through centrifugation for 10 min at 10,000 rpm under 4 °C. The resulting clear supernatant was pooled, representing the cell free extract and stored at -20 °C for further work (Selinheimo *et al*., 2006).

2.4. Enzyme assay

Tyrosinase activity was measured with 2 mL L-tyrosine as the substrate (Ikehata and Nicell, 2000). Activity assays were proceeding in 50 M potassium phosphate buffer (pH 7.0) at 25 °C. The crude tyrosinase extract (1 mL) was added to reaction mixture. After incubation for 10 min at temperature of 25 °C, the absorbance was assayed spectrophotometrically at 505 nm. One activity unit was expressed as the amount of enzyme oxidizing 1 µmol of the L-tyrosine using the standard assay conditions (Selinheimo *et al*., 2009).

2.5. Protein determination

The enzyme protein content was determined by the method of Lowry *et al*. (1951).

2.6. Enzyme purification

Tyrosinase purification was carried out according to Munjal and Sawhney (2002). Crude culture supernatant was resuspended in 50 mM of Tris-HCl buffer (pH 7.5). NH_4 $(SO_4)_2$ was added to the cell free extract to different 40-90% saturations with gentle stirring for 1 h and centrifuged for 30 min at 10,000 g. The precipitate was dialyzed overnight in dialysis bag against 50 mM Tris-buffer (pH 7.5) prepared with 0.15 M NaCl.

The concentrated enzyme preparation was deposited onto a DEAE-cellulose column with flow rate: 17.5 by 1.6 cm, 1 mL min^{-1}, pre-equilibrated with Tris-HCl buffer (100 mM, pH 6.5) and bound protein was then eluted with Tris-buffer (100 mM, pH 6.5) supplemented with 0.1-0.5 M NaCl gradient. Active fractions exhibiting tyrosinase activity were collected, dialyzed and equilibrated with the same buffer prepared with Tris-buffer (100 mM, pH 6.5). The active fractions with the highest enzyme activity were separated by loading onto a Phenyl-Sepharose column, pre-equilibrated with Tris-buffer (100 mM, pH 6.5) and later used for assay.

2.7. SDS-PAGE of purified enzyme

The molecular homogeneity of the purified tyrosinase was detected by one dimensional SDS-PAGE and gel was stained with Coomassie blue (Zaidi *et al*., 2014).

2.8. Tyrosinase induction

A variety of chemical compounds was examined for the substrate-specificity of tyrosinase from *T. reesei* and *T. harzianum*. Guaiacol, catechol, vanillin, caffeic acid, syringaldazine and *P*-coumaric acid were investigated for their capacity to induce tyrosinase activity. The compounds were sterilized by filtration using a Millipore membrane (0.45µm) and added aseptically into flasks. The concentration of the inducers was 0.1, 0.5 and 1 mM. One control was used without the addition of any inducer compound.

2.9. Tyrosinase enzyme inhibition

The inhibition of tyrosinase by kojic acid, cinnamic acid, sodium azide, benzaldehyde and potassium cyanide was analyzed. Putative inhibitors were simultaneously dissolved with substrate in Na phosphate buffer (50 M, pH 7.0). The inhibitory effect was carried out after 10 min incubation at 37 °C. The residual activity of tyrosinase was estimated using assay methods described before (Duarte *et al*., 2012).

2.10. Statistical analysis

All experiments were performed in three replicates and presented as their mean values with their SD, standard deviation.

3. Results

The results of tyrosinase purification are summarized in Table 1. The purification procedure included NH_4 $(SO_4)_2$ precipitation, 85%, followed by DEAE-cellulose, and phenyl Sepharose. The purification of tyrosinase demonstrated a specific activity of 69.39 and 65.11 U mg^{-1} protein from *T. reesei* and *T. harzianum*, respectively. The obtained values of the purification fold were 21.09 and 14.93 for the two tested fungal species mentioned previously in the same order. Subsequently, tyrosinase purification to homogeneity was detected by SDS-PAGE analysis, which showed a single band of 45 and 65 kDa (Figure 1) for *T. reesei* and *T. harzianum*, respectively.

Table 1. Overall purification profile of tyrosinase from two *Trichoderma* sp.

Fungi	Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Trichoderma reesei	Crude extract	45.6	150.2	3.29	1	100
	Amm. Sulphate	16.7	127.5	7.63	2.32	84.89
	DEAE-Cellulose	0.985	30.2	30.66	9.32	20.11
	Phenyl Sepharose	0.183	12.7	69.39	21.09	8.46
Trichoderma harzianum	Crude extract	55.4	240.2	4.36	1	100
	Amm. Sulphate	18.9	139.1	7.36	1.69	57.91
	DEAE-Cellulose	0.970	40.5	41.75	9.58	16.86
	Phenyl Sepharose	0.235	15.3	65.11	14.93	6.37

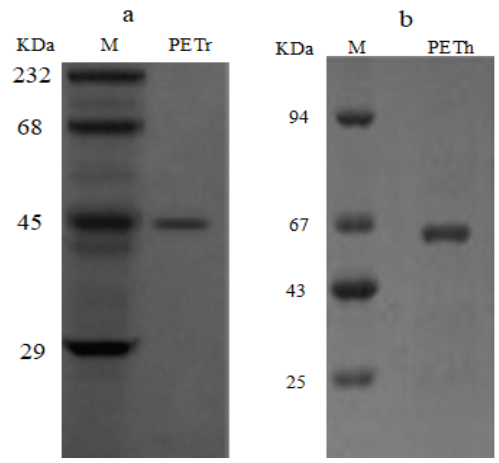

Figure 1. SDS-PAGE pattern of the purified tyrosinase obtained from *T. reesei* and *T. harzianum*. Lane M: molecular mass of marker protein; Lane PETr: purified tyrosinase from *T. reesei* (a) and Lane PETh: purified tyrosinase from *T. harzianum* (b).

The effects of various inducer compounds, monophenolic and diphenolic substrates, on tyrosinase enzyme activity were studied. Control media, without the inducer factor, were considered as 100%. The two *Trichoderma* species showed a dose-dependent response of tyrosinase activity toward all of the examined inducers. The higher the inducer concentration, the higher the induction effect on tyrosinase biosynthesis investigated. All of the inducers clearly pronounced tyrosinase activity. Among the studied diphenolic inducers, caffeic acid at 1mM was more efficient than the other tested inducers by 227% increase in tyrosinase activity in case of *T. reesei* and 184% in case of *T. harzianum*. However, *P*-coumaric acid at 1mM showed the strongest induction effects on *T. reesei*-tyrosinase (254%) and *T. harzianum*-tyrosinase (218%) among the tested monophenolic substrates. It was observed that guaiacol, catechol, vanillin and syringaldazine were less efficient inducer substrate for *T. reesei* tyrosinase and increased the percentage of tyrosinase activity, providing 180, 119, 155 and 181%, respectively (Table 2). Meanwhile, tyrosinase from *T. harzianum* showed less response towards guaiacol (122%), catechol (114%), vanillin (103%), and syringaldazine (137%).

Table 2. Effect of different monophenolic and diphenolic inducers on tyrosinase activity.

Fungi	*Trichoderma reesei*		*Trichoderma harzianum*	
	Activity (U ml^{-1})	%	Activity (U/mg)	%
Control	12.7	100	15.3	100
Guaiacol				
0.1 mM	20.0± 0. 4	157±2.1	17.4±0.6	133±2.9
0.5 mM	22.5±0.5	177±2.3	18.1±0.5	118±1.9
1 mM	22.9±0.7	180±2.2	18.8± 0.6	122±1.8
Catechol				
0.1 mM	11.5±0.3	91.6±1.7	15.0±0.6	98.0±1.9
0.5 mM	14.0± 0.8	11.7±0.3	16.1±0.5	105±1.1
1 mM	15.1±0.5	119±1.6	17.4±0.7	114±1.5
Vanillin				
0.1 mM	18.5±0.6	146±0.5	14.8±0.3	97.0±1.4
0.5 mM	19.5±0.5	154±1.7	15.3±0.5	100
1 mM	19.7±0.4	155±1.9	15.0± 0.5	103±1.5
Caffeic acid				
0.1 mM	28.3±0.4	222±2.5	25.5±0.6	167±1.8
0.5 mM	28.7±0.6	225±2.7	27.8±0.5	182±2.0
1 mM	28.9±0.4	227±2.8	28.1±0.6	184±1.7
Syringaldazine				
0.1 mM	22.0±0.5	173±1.4	19.2±0.6	125±1.8
0.5 mM	22.5±0.4	177±1.3	20.1±0.5	131±1.8
1 mM	23.1±0.7	181±1.8	21.0±0.4	137±1.9
***P*-Coumaric acid**				
0.1 mM	31.2±0.8	210±2.3	27.5±0.6	197±2.4
0.5 mM	37.2±0.7	234±2.7	30.1±0.8	207±2.8
1 mM	39.4±0.9±	254±2.8	35.2±0.9	218±2.5

Several compounds may elicit a negative effect on tyrosinase activity. Tyrosinase enzyme activity was assayed in the existence of kojic acid, cinnamic acid, sodium azide, benzaldehyde and potassium cyanide at pH 5.0. The type of potential inhibition was investigated in the presence of monophenolic substrate like *p*-coumaric acid and diphenolic substrate, caffeic acid (Table 3) according to the above determination assay. A control sample was used without the addition of any inhibitor compounds. KCN was the most powerful inhibitor for tyrosinase enzyme from *T. reesei* and *T. harzianum*-monophenolase activity with 47.5 and 49.0%, respectively. In addition, sodium azide and cinnamic acid also showed significant inhibition toward tyrosinase enzyme from *T. reesei* and *T. harzianum* with 63.2, 74.1 and 60.2, 77.5% residual activity, respectively. Furthermore, the other remaining

substances had less significant inhibitory effect on the monophenolase activity of tyrosinase.

Under the same tested conditions, the response of the diphenolase activity of tyrosinase derived from the tested *Trichoderma* sp. toward the tested putative inhibitors was investigated. Kojic acid was the strongest inhibitor on the diphenolase activity of *Trichoderma*-tyrosinase, followed by potassium cyanide, sodium azide, cinnamic acid and benzaldehyde.

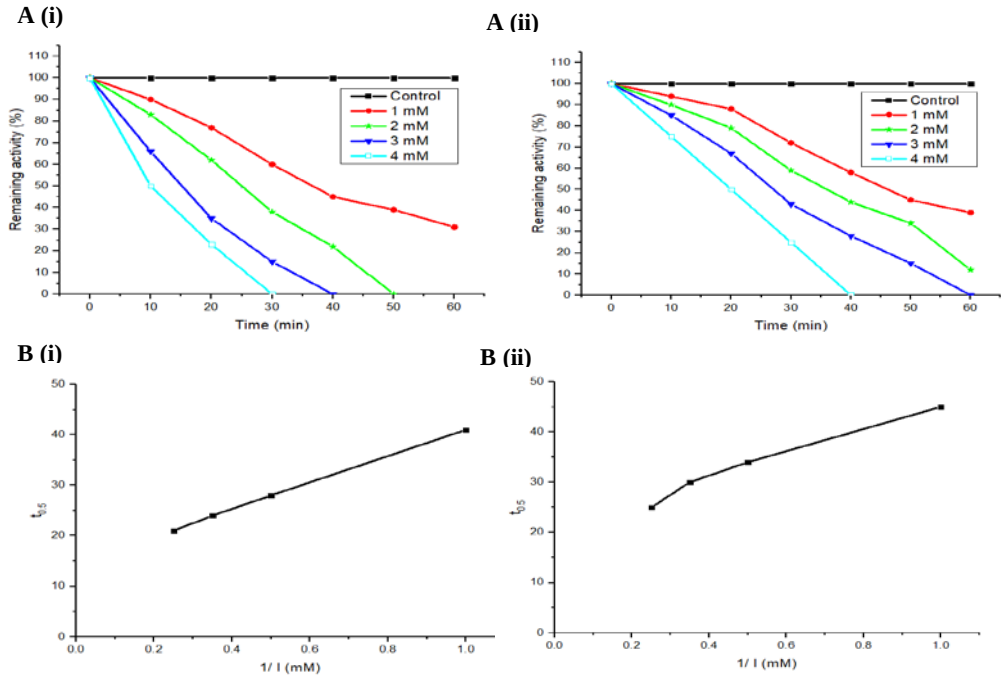

Figure 2. A:.Inactivation of monophenolase activity of tyrosinase produced by *T. reesei* (i) and *T. harzianum* (ii) using potassium cyanide, **B**: relation between $t_{0.5}$ and reciprocal of inhibitor concentration by *T. reesei* (i) and *T. harzianum* (ii).

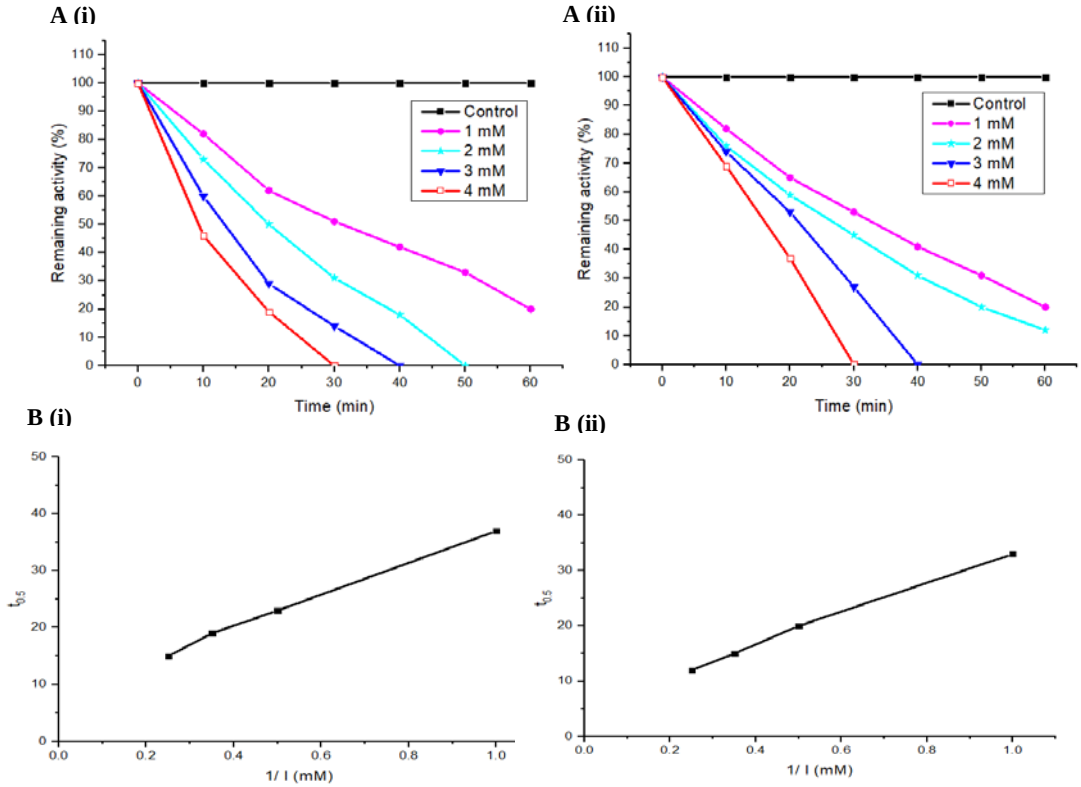

Figure 3. A: Inactivation of diphenolase activity of tyrosinase produced by *T. reesei* (i) and *T. harzianum* (ii) using kojic acid, **B**: relation between $t_{0.5}$ and reciprocal of inhibitor concentration by *T. reesei* (i) and *T. harzianum* (ii).

Table 3.Influence of different inhibitors on mono- and di-phenolase activity of tyrosinase enzyme obtained from two *Trichoderma* sp.

	Residual activity (%) towards *p*-coumaric acid	
	Trichoderma reesei	*Trichoderma harzianum*
Control	100	100
Kojic acid	59.3±1.6	59.1±1.2
Cinnamic acid	74.1±1.5	77.5±1.1
Sodium azide	63.2±1.0	60.2±1.0
Benzaldehyde	80.2±0.9	82.4±1.1
Potassium cyanide	52.5±0.7	51.0±0.8
	Residual activity (%) towards caffeic acid	
	Trichoderma reesei	*Trichoderma harzianum*
Control	100	100
Kojic acid	50.2±0.9	54.5±0.8
Cinnamic acid	79.7±1.0	81.3±1.0
Sodium azide	75.9±1.2	67.5±1.1
Benzaldehyde	99.4±1.5	99.9±1.4
Potassium cyanide	61.5±0.9	57.1±0.9

The rate of tyrosinase activity followed a pseudo-first-order reaction kinetics under the conditions of the inactivation toward mono- and di-phenolase activity by potassium cyanide (Figure 2 A,B) and kojic acid (Figure 3 A,B), respectively. Furthermore, it was observed that the rate of tyrosinase activity was proportional to the inhibitor concentration (Figure 2A, 3A) during the inactivation treatment. The rate constant for potassium cyanide inactivation of tyrosinase was calculated from the slope (Figure2B) as 0.02 and 0.03 μM^{-1} min^{-1}, from *T. reesei* and *T. harzianum*, respectively. However, the rate constant for tyrosinase inhibition from *T. reesei* and *T. harzianum* using caffeic acid was 0.027 and 0.033 μM^{-1} min^{-1} as calculated from the slope of figure 3B, respectively.

4. Discussions

In the present investigation *Trichoderma reesei* and *Trichoderma harzianum* produced appreciable activities of tyrosinase. Well-characterized extracellular tyrosinases were isolated from the fungal strains *T. reesei* (Selinheimo *et al*., 2006) and *Amylomyces rousii* (Montiel *et al*., 2004).

The purification steps included ammonium sulphate (85%), DEAE-cellulose, and phenyl Sepharose. Purified tyrosinase exhibited a final specific activity 69.39 and 65.11 U/mg protein and purification fold of 21.09 and 14.93 for *T. reesei* and *T. harzianum*, respectively. The purity of tyrosinase derived from *T. reesei* and *T. harzianum* was determined by SDS-PAGE analysis, which showed a single band of 45 kDa and 65 KDa (Selinheimo *et al*., 2006; Kawamura-Konishi *et al*., 2007).

The purified tyrosinases were tested in terms of substrate affinity and inhibition. Six compounds were investigated for their capacity to increase tyrosinase enzyme activity; guaiacol, catechol, vanillin, caffeic acid, syringaldazine and *P*-coumaric acid. The control without any inducer showed a low tyrosinase activity. Caffeic acid exhibited the most inductive effect on diphenolase activity of tyrosinase enzyme. However, the other reagents have less inductive effect on tyrosinase activity. In addition, *P*-coumaric acid as monophenolic inducer substrate displayed the highest inductive effect on tyrosinase from the tested two fungal strains. It was found that tyrosinase enzyme exhibited a more activity with increasing the reagents concentration in the culture medium. Tyrosinase enzyme obtained from *T. reesei* showed a remarkable difference in the enzyme–substrate specificity (Selinheimo *et al*., 2009; Fan and Flurkey, 2004). This may be attributed to the variation in binding capability of different substrates toward the binuclear-copper site of tyrosinase (Lim *et al*., 1999; Kubo *et al*., 2004; Selinheimo *et al*., 2009).

Tyrosinase-catalyzed browning reactions of vegetables and fruits are accompanying with shortening of shelf life. Therefore, inhibition of tyrosinase is of great benefit. The inhibition of tyrosinase by cinnamic acid, kojic acid, sodium azide, benzaldehyde and potassium cyanide was analyzed in the presence of monophenolic substrate (*p*-coumaric acid) and diphenolic substrate (caffeic acid). The reaction mixture includes1.0 mM inhibitor and 1.0 mM substrate in phosphate buffer (0.1 M, pH 5.0) and incubated at 25 ºC. Under the tested conditions, the inhibitory action of the investigated inhibitors on the monophenolase activity of tyrosinase from *Trichoderma* sp. showed the subsequent order: KCN> kojic acid >Na azide >cinnamic acid > benzaldehyde. However, the effect of the tested inhibitors on the diphenolase activity was ranking as follows: kojic acid > potassium cyanide> sodium azide >cinnamic acid > benzaldehyde.

The inhibitory effect on tyrosinase activity can be speculated with relation to three possible reasons. First, the length of the lag period correlated to the oxidation reaction catalyzed by tyrosinase was significantly extended by inhibitor molecules (Selinheimo *et al*., 2009). Second, mono- and di-phenolase activity of tyrosinase showed various responses to kojic acid and potassium cyanide as inhibitor. Third, phenolic substrates were variably bound to the tyrosinase-active site. The active site of tyrosinase showed that kojic acid binds to CuB while monophenols bind to CuA (Sendovski *et al*. 2011). Indeed, both kojic acid and caffeic acid contest to CuB on tyrosinase active site. On the other hand, the CuA on tyrosinase active site was occupied by *p*-coumaric acid (Selinheimo *et al*., 2009).

The inhibition of tyrosinase activity was further analyzed with potassium cyanide as monophenolase tyrosinase inhibitors. The activity rate for two *Trichoderma* sp. was determined, with the data plotted as the tyrosinase residual activity versus incubation time. The rate of tyrosinase activity exhibited a pseudo-first-order reaction kinetics and was with linear proportion to the inhibitor concentration.

5. Conclusions

Till now there are no sufficient studies concerning inducers and inhibitors of fungal tyrosinase, accordingly our main purpose in this research aimed to determine the response of tyrosinase from two *Trichoderma* sp. toward various putative inducer substrates and inhibitors. Extracellular tyrosinase was purified by ammonium sulphate precipitation (85%), DEAE-cellulose and phenyl Sepharose from *T. reesei* and *T. harzianum*. SDS-PAGE confirmed the purity of tyrosinase produced by *T. reesei* and *T. harzianum* to homogeneity, exhibiting a single band

of 45 and 65 kDa, respectively. The tested inducer substrates showed a concentration dependent on tyrosinase activity, especially monophenolic substrate as *p*-coumaric acid and diphenolic substrate as caffeic acid as diphenolic substrate. Among the tested inhibitors, KCN and kojic acid had the highest inhibitory effect against the tyrosinase activities from the two investigated *Trichoderma*; monophenolase and diphenolase activities, respectively. The remaining enzyme activity was proportional to the inhibitor concentration with a pseudo-first-order reaction kinetics. Therefore, inducers can be used for increasing the efficiency of tyrosinase.

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