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Research article

Purification, characterization and medicinal application of tyrosinase extracted from *Saccharomyces cerevisiae*

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Abstract

Extracellular tyrosinase extracted from *Saccharomyces cerevisiae* was purified, characterized and assayed as medicinal agent. The crude enzyme activity was 7.99 U/ml and specific activity was 0.84 U/mg. The complete purification protocol was done. The final result showed one peak of pure tyrosinase with activity of 1.08 U/ml, specific activity of 2.31 U/mg, purification fold of 31.25 and recovery of 22 %. The molecular mass of the enzyme was approximately 40 KDa. Characterization of the pure enzyme indicated that the optimum enzyme concentration was 4 mg protein/ml, however, optimum substrate [tyrosine] concentration was 2.2 mg/100 ml. The k_m and V_{max} values were 2.56 mg/ml and 20 U/ml, respectively. PH 9 proved to be the optimum value, while 35 °Cwas the optimum temperature. $CuSO_4$>$MgCl_2$>$ZnSO_4$ enhanced the enzyme activity; however, $HgSO_4$ completely inhibited it. Assaying the enzyme inhibitors, declared that the metal chelating compound EDTA completely inhibited the enzyme activity which showed clearly that it is a metaloprotein. Medicinal applications of tyrosinase indicated that it exert weak antimicrobial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *E. coli* and *Staphylococus aureus*, but not have any antifungal activity. The enzyme proved to have antioxidant activity 50% of glutathione peroxidase. It also increased the viability of normal skin melanocyte cell line [MFB-4] before and after UV-irradiation indicated its protective and healing effect against UV-rays. The enzyme also reducing the viability of breast carcinoma cell line [MCF-7] after and before UV exposure declared that it may have anticancer activity.

Introduction

Tyrosinase [EC 1.14.18.1] is a copper containing monooxygenase catalyzing the o-hydroxylation of monophenols to the corresponding catechols [monophenolase or cresolase activity] and the oxidation of monophenols to the corresponding o-quinones [diphenolase or catecholase activity]. It is involved in the biosynthesis of melanin and catalyses the ortho-hydroxylation of tyrosine [monophenol] to 3, 4-dihydroxypheny-lalanine or DOPA [o-diphenol] and the oxidation of DOPA to dopaquinone [o-quinone]. This o-quinone can then be transformed into melanin pigments through series of non-enzymatic and enzymatic reactions [1-2]. The mechanism by which an oxygen atom is transferred to the phenolic substrate is suggested to begin with either an oxodicopper [III] intermediate or a peroxodicopper [II] intermediate [3]. Melanogenesis is a biosynthetic pathway for the formation of the brown pigment melanin in the human skin. Tyrosinases are the key enzymes catalyzing the different steps in melanogenesis. Since that time, the role of trosinases in melanin production and its melanogenic properties has high focus [3]. There are two types of melanin pigments which produced by the melanocytes namely [eumelanin] black or brown melanin and [pheomelanin] red or yellow melanin [4]. Each type of melanin production depends on the melanocytes functioning. Individuals with dark skin are genetically programmed to produce continuously high levels of melanin even without exposure to UV light. In fair skin colour, melanosomes packed themselves in the form of membrane-bound organelles [4-6]. Tyrosine is first oxidized to dopaquinone which either react with cysteine to give a precursor or reddish brown pheomelanin or cyclise to give a dihydroxyindole precursor of black or brown eumelanin. Over activity of tyrosinase results in hyperpigmentation of the skin while weak activity lead to a vitiligo depigmented spots in the skin and hair whitening [7]. Many biotechnological applications of monophenolase and diphenolase activities of tyrosinase was used such as detoxification of phenol-containing waste waters and soil [8], production of L-DOPA, dopamine for the treatment of Parkinson's disease, used as a marker in melanoma disease [9], activation of prodrugs [10], modification of food via crosslinking affects protection against radiation [UV and

γ rays], drug carriers, antioxidants, antiviral and immunogen factors [11]. Tyrosinases play an important role in regulation of oxidation-reduction potential and the wound healing system in plant and animals [12]. Tyrosinase activity has an essential role in some plant–derived food products such as tea, coffee, raising and cocoa, where it produces distinct organoleptic properties [13]. Tyrosinase in fungi has been induced only under stress conditions. This indicated that tyrosnases improve the survival and protect fungi by producing melanin [14]. The information on the role of the tyrosinases in yeasts has been limited to date. However, melanin plays a role in the protection of cell wall after any physical damage [15].It is also important for pigmentation and it essential factors in wound healing and primary immune response. It binds toxic heavy metals in the cell [15] and provides protection against oxidants, heat, enzymatic hydrolysis and antimicrobial compounds [16]. Yeasts are eukaryotic microorganism whose genome has been extensively studied and some have been sequenced. Yeasts are relatively easy to grow under laboratory conditions. They have membrane bound enzymes and organelles, nuclear DNA, transcription mechanisms and cytokins, which are similar to those found in higher eukaryotes. Yeasts also have many extracellular proteases and pheromones. Yeast culture media are different. With the various applications required such as synthesis of single cell protein, medicinal applications and bioremediation. Yeast culture media should be including yeast extract, peptone and dextrose or glucose. Knowledge of tyrosinases in yeast is still limited and the researches have been resulted in very low productivity of the enzyme, so in this study the production, purification and characterization of tyropsinase from *Saccharomyces cerevisiae* was carried out. Several medicinal applications of tyrosinase e.g. antimicrobial activity, antioxidant activity, antitumor activity and melanogenic action were also studied.

Materials and methods

Test organism

Saccharomyces cerevisiae 006-001 was purchased from the culture collection of Reginal Centre for Mycology and Soil, Al-Azhar University.

Cultivation and maintenance of *S. cerevisae*

The production medium was composed of [g/l]; Glucose [5], peptone [1], yeast extract [1] and $CuSO_4$ [0.5]. The inoculated culture was incubated at 30 ° C under static conditions for 10 days. The culture was then filtrated aseptically through whatman filter paper No 1. The resulting colonies were picked on slants containing Sabouraud agar for maintenance. The filtrate was then rotated in cooling centrifuge [Hettich zentrifugen. Universal 16/16R] at 5000 rpm for 15 min, to remove any spores and other particles and the centrifugate was collected. Protein content and tyrosinase activity were determined in the centrifuge [crude enzyme].

Assay of tyrosinase activity

Tyrosinase activity was assayed using 15 mM L-DOPA and 2 mM L. tyrosine as substrates [17]. The reaction mixture was carried out in 0.1 M sodium phosphate buffer [pH 7.0] at 25°C. The reaction was initiated by addition of 10 uM of enzyme to the substrate solution. Dopachrome formation was monitored at 470 nm. The enzyme activity unit was expressed as the amount of enzyme required to oxidize 1 µM of the substrate in one min under standard assay conditions.

Specific activity [U/mg] = enzyme activity [U/ml]/protein [mg].

Determination of protein

Protein content in the reaction mixture was determined according to the method described by Lowry *et al.*, [18].

Purification of tyrosinase

The yeast culture was centrifuged at 5,000 rpm for 15 min under cold conditions, and the supernatant obtained after centrifugation was used as a source of crude enzyme.

Ammonium sulphate preparation

The partial purification of tyrosinase was carried out by using ammonium sulphate precipitation [20-80%]. The mixture was centrifuged, and precipitated protein was dialyzed against 20 mM sodium phosphate buffer [pH 7.4]. The protein content and tyrosinase activity were estimated. The mixture was then loaded on to a sephadex G-100 column [1.3×90 cm] equilibrated with sodium phosphate buffer 50 mM [pH 7.4]. The column was washed with the same buffer, and the enzyme was eluted with 0.05 to 0.25 M NaCl linear gradient. Fractions containing tyrosinase activity were pooled and dialyzed against 20 mM sodium phosphate buffer [pH 7.4].

Molecular mass determination using SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis [SDS-PAGE] was used. The molecular mass of purified enzyme was determined by calculating the relative mobility of standard protein markers such as, myosin rabbit muscle [205 kDa], phosphorylase b [97.4 kDa], bovine serum albumin [66.0 kDa], ovalbumin [43.0 kDa], and carbonic anhydrase [29.0 kDa].

Factors affecting the enzyme activity

Effect of enzyme concentration on tyrosinase activity

Four different concentrations of tyrosinase [3, 4, 5, and 6.5 mg protein/ml] were used. The reaction was performed at 35°C for 30 minutes.

Effect of substrate concentration on tyrosinase activity and enzyme kinetics

Six different concentrations of L-tyrosine [0.45, 0.9, 1.8, 2, 2.2, 2.4 mg/100 ml] were used in this study. The Km and Vmax were calculated using [Michaelis-Menten curves by Graphpad software, Juc, San Diego, GA, USA],

Effect of pH on tyrosinase activity

To study the effect of different pH [4-10] on tyrosinase activity, three different buffer systems were used. These were acetate buffer for pH 4 & 5, phosphate buffer for pH 6 & 7 & 8 and tris HCL for pH 9 & 10. The reaction mixture was obtained under the optimum enzyme and substrate concentrations. Incubation was conducted at 35°C for 30 minutes. The protein and tyrosinase activity were determined in each case.

Effect of temperature on tyrosinase activity

The reaction mixture was incubated at each of 8 different temperatures [0, 20, 30, 40, 45, 55, 60 and 65°C]. The optimum temperature for enzyme activity was estimated after determination of tyrosinase activity.

Effect of metal ions and inhibitors on tyrosinase activity

The effect of different metal ions [NaCl, $CaCl_2$, $CuSO_4$, $MgCl_2$, $ZnSO_4$, $CoCl_2$, $HgCl_2$ and $FeCl_3$] on tyrosinase activity was studied under optimum conditions of enzyme activity. Some inhibitors [cysteine, thiourea, kojic acid, EDTA and ascorbic acid] were studied by incubating the enzyme for 15 min at optimum temperature in presence 1.0 mM of each inhibitor.

Substrate specificity of the tyrosinase

Different substrates such as L-tyrosine, catechol, L-DOPA, p-cresol, phenol and pyrogallol were assayed with tyrosinase. The protein and tyrosinase activity were determined in each case.

Antimicrobial activity of tyrosinase

The antimicrobial activity of tyrosinase was assayed against *Salmonella typhimurium* [ATCC 14028], *Saphylococcus aureus* subsp. *aureus*[ATCC 25923], *Staphlococcus aureus* [MRSA] [ATCC 43300], *Bacillus cereus* [ATCC 33018], *Bacillus subtilis* subsp *spizizenii* [ATCC 6633], *Listeriamonocyogenes* [ATCC 7644], *Listeria innocua* [ATCC 33090], *Escherichia coli* [ATCC 11775], *Pseudomonas aerugmosa* [ATCC 10145] *Candida albicans* [ATCC 26555] and *Aspergillus niger* [nrrl 326] [19]. The susceptibility was assayed using CLCI, M44A on Mueller Hinton agar plate.

Antioxidant activity of tyrosinase

The antioxidant activity of tyrosinase was carried out according to Paglia and Valentine [20]. The reaction mixture contains one ml phosphate buffer; 0.1 ml NADP; 0.01 ml sample an o.1 ml H_2O_2 then the absorbance is read at 340 nm over a period of 3 min.

Cytotoxicity assay of tyrosinase enzyme

Potential antitumor activity of tyrosinase was assayed using the method of Skehan *et al.* [21] as follows: two cell lines; human skin [HFB-4] and breast carcinoma cell line [MCF_7] were planted in 96-multi well plate [10^4cell/well] for 24 h before treatment with tyrosinase to allow attachment of cells to the wall of the plate. Different concentrations of enzyme under test [0.0, 6.25, 12.5., 25, 50 and 100 U/ml] were added to monolayer cells. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the enzyme for 48 h at 37°C and 5% CO_2 atmosphere. After 48 h, cells were fixed, washed and stained with sulfo–rhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer. Colour intensity was measured with an ELISA reader. The relation between surviving cells [viability %] and enzyme concentrations were plotted to get the survival curve of each tumor cell lines.

Results and discussion

Assay of tyrosinase enzyme extracted from *S. cerevisiae*

The crude extracellular extract from *S. cerevisiae* was found to have tyrosinase activity of 9.9 U/ml and specific activity of 0.63 U/mg. Jadhav and Govindwar [22] studied the enzymes in *S. cerevisiae* responsible for biodegradation of methyl red control cells and obtained after decolourization in plain distilled water [pH 6.5 and pH 9]. They showed different levels of tyrosinase, lignin peroxidase, NADH-DCIP reductase, azoreductase, and aminopyrine N-demethylase activities. A significant increase in the activities of tyrosinase, lignin peroxidase and NADH-DCIP reductase were observed in the cells obtained at pH 9. It appears that Ascomycetous fungi have secreted tyrosinases such as yeast, *Neorospora cressa* and *Magnapothe grisae*. Tyrosinases have been produced heterogeneously in *Saccharomyces cerevisiae* [23-26]. Roy *et al.* [27] observed few black colonies on the selection plates of *S. cerevisiae*. These colonies were found to be tyrosinase producer budding yeast and able to produce melanin and identified as novel *Exophiala jeanselmei*. In *Streptomyces* sp. and yeast sp. Tyrosinases are secreted in the surrounding medium and catalyzes the melanin formation [28-29]. Tyrosinase which isolated from different sources, mushroom, bananas, potatos and yeasts showed that the most effective source of tyrosinase was *Agaricus bisporus* [30]. The yeast *Phialophoraseansel*

mei produce tyrosinase that forms melanin contaminated polysaccharide in the culture media [31-34]. Tyrosinase activity from *S. cerevisiae* was determined in reaction mixtures containing 1% catechol in 0.1 M phosphate buffer [pH 7.4]. The formation of catechol quinine was measured at 410 ug [Bioplass, Biotede, Biodum 2015].

Purification of extracellular tyrosinase from *S. cerevisiae*

Tyrosinase is an omnipresent type three copper enzyme participating in many essential biological functions. Purification and understanding characterization of the enzyme is essential for developing its important applications. Table 1 show that the crude extract had tyrosinase activity 7.99 U/ml; protein, 9.55 mg and specific activity 0.84 U/mg. Protein precipitation performed by different $[NH_4]_2SO_4$ concentrations indicating that saturation attained at 80 % $[NH_4]_2SO_4$. After desalting of the enzyme by dialysis overnight, the tyrosinase activity reached 6.26 U/ml with specific activity 2.50 U/mg. The purification fold was 2.98 with recovery of 78 %.The enzyme extract was subjected to gel filtration chromatography using sephadex G-100 as second step of enzyme purification. Two activity peaks were resulted; the first peak included fractions 9, 10 and 11 with maxima at peak 10 where the enzyme activity was 7.3 U/ml, specific activity 2.28 U/mg, with purification fold of 1.14 and recovery of 79.58.The second peak constituted fractions 12 an 13 with maxima at peak 12 where the tyrosinase activity was 2.67 U/ml with specific activity of 0.35 U/mg, purification fold of 0.4 and recovery of 33.50.The composite sample containing fractions from 9-13 resulted from sephadex G-100 were subjected to cation exchange chromatography by DEAE- cellulose column. The enzyme showed one pure peak with enzyme activity, protein, specific activity, purification fold and recovery recorded values of 1.8 U/ml, 1.08 mg, 2.31 U/mg, 31.25 and 22 %, respectively [Table 1].

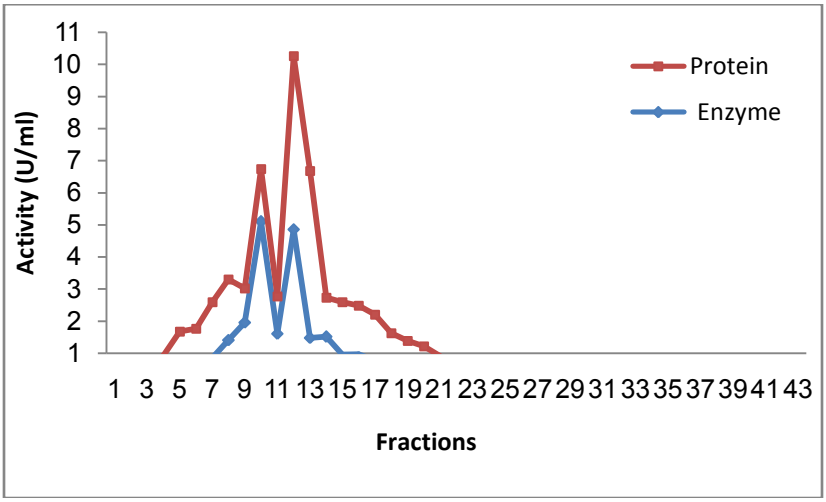

Figure 1. Elution Profile of Sephadex G-100 fraction ion exchange Column Chromatography. An Aliquote of Each Fraction was assayed for Protein Content and Tyrosinase Activity. Tyrosinase Eluted with Linear Gradient of Potassium Phosphate Buffer at 0-100 mM.

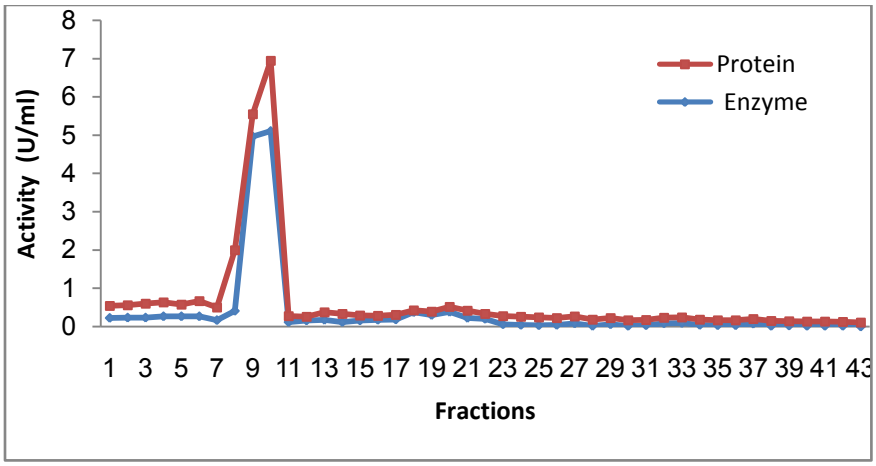

Figure 2. Chromatographic Profile from DEAE-Cellulose column for Tyrosinase of *S. cerevisiae*.

Table 1. Full purification protocol of *S. cerevisiae* tyrosinase.

Purification Step		Total Activity [U/ml]	Total Protein [mg]	Specific Activity [U/mg]	Purification Fold	Recovery [%]
Crude Extract		7.99± .031	9.55± .033	0.84	100	100
$[NH_4]_2SO_4$		6.26± .031	2.50± .021	2.5	2.98	78
Sephadex G-100	Peak 1	7.30± .011	3.20± .031	2.28	1.14	79.58
	Peak 2	2.67± .032	7.6± .040	0.35	0.4	33.50
DEAE-Cellulose		1.8± .011	1.08± .012	2.31	31.25	22

P> 0.05.

Selinheime *et al.* [35] purified expressed tyrosinase in *S. cerevisiae* with three steps of purification consisting of $[NH_4]_2SO_4$ precipitation, desalting, gel filtration, cation exchange chromatograph and size exclusion chromatography with recovery of 20 % of the crude preparation. Roy *et al.* [27] showed that the crude extract or dialysate of tyrosinase from *Exophiala jeanselmei* after $[NH_4]_2SO_4$ precipitation and 7.5 % or 10 % polyacrylamide gel containing SDS exhibit two bands of approximately molecular mass of 100 KDa and 120 KDa when stained with DOPA [0.8 mg/ml] for tyrosinase activity. Zaidi *et al.* [3] purified tyrosinase from *Agaricus bispora* by $[NH_4]_2SO_4$ precipitation, gel filtration by sephadex G-100 chromatography and ion exchange chromatography on DEAE cellulose chromatography. The enzyme was purified to 36.36 fold with 26.6 % recovery of crude preparation and specific activity of 52.9 U/mg.

Molecular mass determination

Discontinuous SDS-PAGE was performed using a mini-protein electrophoresis unit [Biorad]. The apparent molecular mass of tyrosinase was determined using the following markers of aprotinin [9 kDa], lysozyme [19 kDa], myoglobin [26 kDa] and carbonic anhydrase [38 kDa], ovalbumin [46 kDa], glutamate dehydrogenase [62 kDa] and bovine serum albumin [91 kDa]. Figure 3 indicated that the enzyme protein reached the full homogeneity in one protein band with apparent molecular mass of approximately 40 kDa. In close correlation with the present results, low molecular weight tyrosinase was reported by Selinleimo *et al.* [36] who found that the apparent molecular mass of purified tyrosinase expressed by *Trichoderma reesei* in *S. cerevisiae* was significantly low and was approximately 43 KDa. The molecular mass of tyrosinase from *Vibrio tyrosiaticus* is of 36 KDa [33], while that of *Neurospora crassa* is 33 KDa [37] and of frog skin tyrosinase is of 50 KDa [38]. *Streptomyces* sp tyrosinases are monomeric protein with relatively low molecular mass of 30 KDa. However, filamentous fungi, have tetrameric tyrosinase as in enzyme extracted from the mushroom *Agaricus bisporus* where the molecular mass is 120 KDa having monomeric isoform with molecular mass of 30 KDa [39]. Similarly, high molecular weight of tyrosinase of 140 KDa was determined using SDSA-PAGE from *Aspergillus flavus* UVFP570 [40].

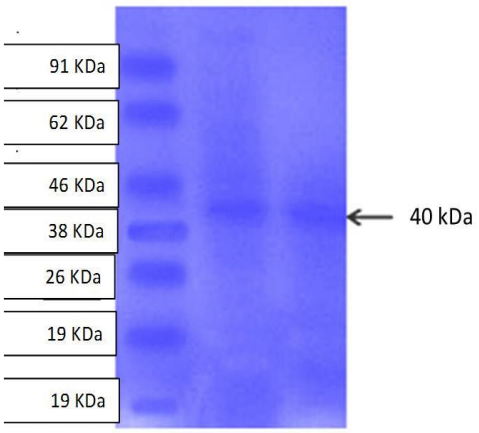

Figure 3. SDS-Polyacrylamide gel electrophoresis of *Saccharomyces cerevisiae* tyrosinase.

Characterization of the purified tyrosinase

Effect of enzyme concentration on purified tyrosinase activity

The activity of the purified enzyme was measured with increasing concentrations of enzyme [3, 4, 5, and 6.5 mg protein/ml] [Table 2]. The data obtained revealed that the optimum enzyme concentration was 4 mg protein/ml, at which, the tyrosinase activity, protein and specific activity were 7.3 U/ml, 0.8 mg and 9.12 U/mg, respectively. At 3.0 mg/ml enzyme concentration, a decrease in enzyme activity of 3 % was attained while at the highest enzyme concentration [6.5 mg/ml] the decrease in enzyme activity was only 4.11 %.

Effect of substrate concentration on tyrosinase activity and kinetics

The activity of the purified enzyme was measured with increasing concentrations of the substrate [tyrosinase] [0.45, 0.9, 1.8, 2.0, 2.2, 2.4 mM] [Table 3]. A hyperbolic relationship between the activity of the tyrosinase and the substrate concentration was obtained.

The enzyme activity increased to reach maximum at 2.2 mM. The enzyme activity, protein and specific activity were 8.6 U/ml, 0.91 mg and 9.5 U/mg, respectively.

Substrate inhibition becomes apparent at the highest concentration [2.4 mM].

Table 2. Effect of enzyme concentration on purified tyrosinase activity.

Enzyme concentration [mg protein /ml]	Enzyme activity[U/ml]	Protein [mg]	Specific activity [U/mg]
3.0	5.1± .033	1.2± .011	4.25
4.0	7.3± .022	0.8± .022	9.12
5.0	7.2± .045	2.2± .031	3.27
6.5	7.0± .031	2.8± .032	2.5

P> 0.05.

Table 3. Effect of substrate concentration on *S. cerevisiae* tyrosinase.

Substrate concentration [mM]	Tyrosinase activity[U/ml]	Protein [mg]	Specific activity [U/mg]
0.45	0.0± .046	0.98± .035	0.0
0.9	4.0± .015	1.02± .015	3.9
1.8	7.0± .050	1.22± .050	5.7
2.0	8.0± .036	0.88± .045	9.1
2.2	8.6± .045	0.91± .011	9.5
2.4	8.5± .011	1.18± .032	7.9

P> 0.05.

Determination of K_m and V_{max}

From Figure 4, it is clear that the maximum velocity [V max] was found to be 20 U/ml. The substrate concentration that coincides with half maximum velocity is called K_m or Menten constant it was of 2.56mM. The low Km indicated high binding affinity of tyrosinase for its substrate. Different k_m values for L-DOPA were 30 mM, for *Trichoderma reesie* tyrosinase, 0.74-1.09 mM for *Neorospora crassa* [41-42], 0.75mM for *Bacillus megaterium* [43], 5.0 mM for *Aspergillus flavus* [44], 5.97 mM for *Streptomyces glaucescens* [45], and 8.7-10 for pin needle tyrosinase [46]. The purified tyrosinase from mushroom *Agaricus bisporus* reported high affinity towards its natural substrate L-DOPA with an apparent k_m value of 0.933 mM.

Substrate specificity of tyrosinase

Tyrosinase activity was assayed in presence of various substrates: L-tyrosine, catechol, L-dopa, p-cresol, Phenol and pyrogalol. The activity was measured with concentration of substrate of 20 mM in 0.1 M sodium Phosphate buffer [pH 7.0] [Table 4].

Table [4] clearly detected that tyrosinase purified from *S. cerevisiae* is non specific protease and showed broad substrate specificity. However, it was highly active on both pyrogallol and L-DOPA, with enzyme activity of 27 and 20 U/ml, specific activity of 15.5 and 12.3 U/mg, respectively. Phenol and p- cresol were poor substrates for tyrosinase. Similarly, Barber *et al.* [15] reported that pyrogallol was a good substrate for tyrosinase activity, whereas tyrosine, catechol, orcinol and phenol were poor substrates. Dopachrome was found to be insignificant. Yamamoto *et al.* [46] found that a tyrosinase activity catalyzing the formation of DOPA from tyrosine was separated from a polyphenol-oxidase activity catalyzing specifically the oxidation of DOPA to dopaquinone. However, Selinheimo *et al.* [35] found that expressed tyrosinase in *Saccharomyces cerevisiae* showed broad substrate specificity and was highly active on tyrosine and L-DOPA. The enzyme has higher affinity for the L-isomer of the substrate than for the corresponding D-isomers. Not only the physiological substrates tyrosine and L-DOPA, but also various other phenols and diphenols are converted by tyrosinases to the corresponding dipheols and quinines.

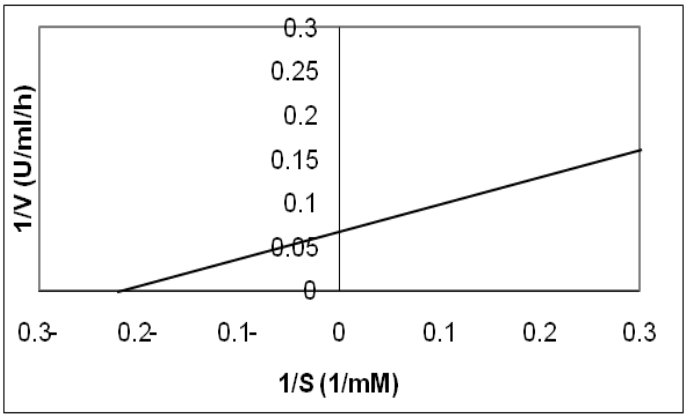

Figure 4. Lineweaver-Burk plot relating the reaction velocity of *S. cerevisiae* tyrosinase to substrate concentration.

Table 4. Substrate specificity of tyrosinase of *S. cerevisiae.*

Substrate	Tyrosinase Activity [U/ml]	Protein [mg]	Specific Activity [U/mg]
L-tyrosine	8± .011	0.92± .021	8.7
Catechol	10± .031	1.10± .014	9.1
L-Dopa	20± .011	1.62± .031	12.3
p-Cresol	3± .033	0.76± .012	4.0
Phenol	2.0± .000	0.69± .031	2.9
Pyrogallol	27± .044	1.74± .011	15.5

p>0.05.

Effect of pH on purified tyrosinase activity

Purified enzyme exhibited a momentous dependency on the pH with optima at pH 9 at which maximum tyrosinase activity [5.2 U/ml] was observed [Table 5]. The enzyme remained active up to pH 10, but 88.8 % of the activity was lost. However, the loss in activity at pH 4.0 reached 75.5% of the optimum value.

Table 5. Effect of pH values on purified tyrosinase of *S. cerevisiae*.

pH	Tyrosinase Activity [U/ml]	Protein [mg]	Specific Activity [U/mg]
4	1.1± .011	1.52± .031	0.73
5	1.4± .015	1.62± .022	0.86
6	2.4± .050	1.41± .014	1.70
7	3.2± .045	1.21± .012	2.60
8	4.0± .050	0.99± .041	4.00
9	4.5± .032	1.87± .015	5.20
10	0.5± .046	0.79± .010	0.63

P> 0.05.

In this connection, all tyrosinases are metaloproteases have a common binuclear type II copper centre [T3Cu] of two copper atoms, each coordinated by three histidine residues, within their active site. The copper pair in the active site binds to atmospheric oxygen to catalyze two enzymatic reactions a] ortho-hydroxylation of monophenol and b] oxidation of o-phenol to o-quinones [54-55]. The catalytic oxidation of a substrate typically involve formation of a reactive intermediate by the reaction of a reduced copper centre with molecular oxygen which may also be incorporated to the substrate [56]. The addition of copper to the growth medium of *Trichoderma ressie* had positive effect on tyrosinase production but not on growth of the fungus. This implies that the higher enzyme yields may have been due to improved folding of the active enzyme in presence of elevated copper concentration [36]. Tyrosinases are known to be copper containing enzyme with possible involvement of systeine and histidine at the active site [57-58].

Effect of some inhibitors on tyrosinase activity

The data indicated that all tested compounds inhibited tyrosinase activity but with different percentage [Table 8]. The metal chelating compound EDTA completely inhibited tyrosinase activity so proved that the enzyme is a metaloprotease. Maximum inhibition was achieved in case of ascorbic acid [40%], followed by cysteine [35%], kojic acid [30%]. However, thiourea and sodium azide led to weak inhibition to tyrosinase activity than the former three compounds with percentages inhibition of 20% and 22% of control value, respectively. In this field, Lerch, 1978 and 1983b reported that thiol group is not essential for tyrosinas activity; Prezioso *et al.* [60] found that phenyl thiourea is specific inhibitor of tyrosinase activity and increase the effectiveness of melanoma drugs. Gasowska *et al.* [61] reported also that thiol group in the phenolic ring inhibited the enzyme. Merdinger *et al.* [62] found that tyrosinase activity extracted from *Pycnoporussanguineus* CCT-4518 was completely inhibited by phentthiourea, salicylhydroxamic acid and partially inhibited by sodium azides.

Medicinal applications of tyrosinase

Antimicrobial activity of tyrosinase

Out of nine tested pathogenic bacterial species, the enzyme could inhibit the growth of four species only, *Bacillus subtilis, Staphylococcus aureus* subsp. *aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, with inhibition zone diameters of 9, 7, 6 and 6 mm, respectively [Table 9]. Tyrosinase showed no antifungal activity either on the filamentous fungal species *Aspergillus niger* or on the unicellular fungal species *Candida albicans*. No available data could be obtained in that field except that of Nosanchuk and Casaevell [63] who reported an antimicrobial action of tyrosinase which contributed to microbial pathogenesis.

Antioxidant activity of tyrosinase

Regarding the antioxidant activity of tyrosinase, the enzyme recorded 50 % activity power out of the control [glutathione peroxidase] value [Figure 5]. In this connection, it was reported that among the primary cause of aging in yeast *Saccharomyces cerevisiae* are free radical oxidative damage telomere corrosion, depletion of stem cells, mitochondrial dysfunction, DNA maturation, genomic instability, epigenetic changes, proteotoxcity and accumulation of extrachromosomal DNA circles [64-70]. Zaidi *et al.* [3] reported that melanin pigments synthesized by tyrosinase have found applications as antioxidant and for protection against radiations, cation exchange, drug carriers, antiviral and immunogen. [71] found that melanin formed from tyrosinase oxidation of tyrosine play an important role in removing the reactive oxygen species in the skin and protect the skin from the dreadful UV-radiation by absorbing UV rays. Tyrosinase from the yeast *Sacchromyces cerevisiae* displayed a high DPPH radical scavenging antioxidant activity in the protein hydrolysate [72].

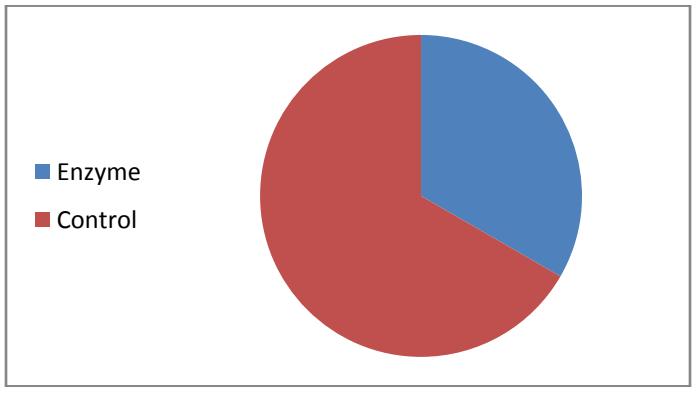

Figure 5. Antioxidant activity of tyrosinase of *S. cerevisiae*.

Table 8. Effect of some inhibitors on tyrosinase activity.

Inhibitor	Enzyme activity [U/ml]	Protein [mg]	Specific activity [U/mg]	Inhibition percentage [%]
Control	4.0± .031	1.2	3.3	---
Cysteine	2.6± .021	1.4	1.8	35
Thiourea	3.2± .044	1.9	1.7	20
Kojic acid	2.8± .032	2.0	1.4	30
Ascorbic acid	2.4± .046	2.1	1.14	40
EDTA	0.00±000	2.0	0	0.0
Sodium azide	3.1±0.44	2.2	1.4	22.5

P> 0.05.

Table 9. Antimicrobial activity of tyrosinase of *S. cerevisiae.*

Microorganisms	Inhibition zone diameter [mm]
Bacillus cereus [ATCC 33018]	0± .000
Bacillus subtilis subsp *spizizenii* [ATCC 6633]	9± .015
Escherichia coli [ATCC 11775]	6± .021
Listeria innocua [ATCC 33090]	0± .000
Listeriamonocyogenes [ATCC 7644]	0± .000
Pseudomonas aeruginosa [ATCC 10145]	6± .032
Salmonella typhimurium [ATCC 14028]	0± .000
Staphylococcus aureus subsp. *Aureus* [ATCC 25923]	7± .034
Staphlococcus aureus [MRSA][ATCC 43300]	0± .000
Aspergillus niger [nrrl 326]	0± .000
Candida albicans [ATCC 26555]	0± .000

P> 0.05.

Effect of tyrosinase on human normal skin melanocytes [HFB-4]

Melanocytes are specialized skin cells that produce protective skin melanin darking pigments to protect skin against UV-radiation. Table [10], indicated generally that the least tyrosinase concentration produced the highest viability of HFB-4 cell lines, however as the enzyme concentration increased the viability of melanocytes decreased to reach the least value at the highest enzyme concentration [100 U/ml] the IC50 was 42.9 U/ml. In Table [10 Column B], when the melanocyte cell lines incubated with enzyme for 48 h then exposed to UV for 1h, the viability percentage of the melanocytes increased indicating high survival capacity of HFB-4 against UV due to the protective action of tyrosinase. The IC_{50} reached 55.2 U/ml. Table [10 Column C], when melanocyte cell lines incubated with tyrosinase with its substrate[tyrosine] for 48h then exposed to UV radiation for 1h, further increase in viability percentage was recorded at all enzyme concentration with IC_{50} value of 38.6 U/ml. This may be due to the formation of melanin which protects the melanocytes against UV irradiation.

Table 10. Viability of human normal skin melanocytes [HFB-4] under different treatments of tyrosinase singly and in combination with UV- irradiation.

Enzyme conc. [U/ml]	Viability [%]				
	A	B	C	D	E
0	100	100	100	100	100
6.25	84.8	98.6	92.1	78.3	82.3
12.5	84.7	82.0	87.1	77.1	76.9
25	71.1	80.2	83.4	69.2	69.0
50	44.5	63.9	81.2	64.4	66.1
100	34.6	41.0	43.4	26.4	13.0
IC_{50} [U/ml]	42.9	55.2	38.57	65.0	46.7

Viability of HFB-4 after exposure [1h] to UV was 11.2 %.A; Human normal skin melanocyte cell lines [HFB-4] + tyrosinase [48 h]. B; HFB-4 cell line + tyrosinase [48 h] then UV [1h]. C; HFB-4 cell line + tyrosinase [48 h] + tyrosine [48 h] then UV. D; HFB-4 cell line + UV [1h] then tyrosinase [48 h]. E; HFB-4 cell line + UV [1h] then tyrosinase + tyrosine [48 h].

These results indicated that tyrosinase not only protective melanocytes from UV-irradiation but also treated the harmful effect of the irradiated skin a phenomenon that may have an important medicinal application. In both cases in table 10 Column D & E when the melanocyte cell lines exposed to UV-irrdiation for 1 h then incubated with either tyrosinase only or tyrosinase with its substrate tyrosine, the viability percentage increased than the cell line exposed to UV only which was 11.21 % this indicate that the enzyme neutralized or treated the harmful effect of UV-exposure. In this connection, melanogenesis is the process of production of melanin by melanocytes within the skin. Melanocytes contain specialized lysosome organelles termed melanosomes which contain tyrosinases that mediate the production of melanin [72-73]. In fungi, tyrosinases are associated with melanin production constituted a mechanism of defense to stress factors such as UV- irradiation, ɣ rays, free radicals, dehydrogenation, extreme temperature and contribute to the fungal cell wall resistance against hydrolytic and lysing enzymes [74]. The degree of malignancy of pigmented cells shown to correlate with tyrosinase activity [75]. Tyrosinase is absent from normal cell, but over expressed in tumor cell. So it provide in built drug delivery mechanism as selective for melanoma tumor [70]. Zaidi *et al.* [3] reported that tyrosinases are involved in several biological activities and defense mechanism especially in melanogenesis. He also found that tyrosinase activity enhanced the antimelanoma effect of the two drug 4-5-cysteaminylphenol and N-acetyl-4-5-cysteaminylpheol in pigmented melanoma.

Effect of tyrosinase on breast carcinoma cell lines (MCF-7)

The data in Table [11], A revealed that the viability of MCF-7 is a function of enzyme concentration. Minimum viability (26.4 %) was obtained at the highest tyrosinase concentration [100 U/ml] the IC_{50} reached 55.0 U/ml. When MCF-7 cell lines incubated with tyrosinase and its substrate for 48 h, the viability of breast cell line decreased with IC_{50} of 30.7 % indicating that the enzyme may had anticancer activity. In this field, melanine pigments resulted from activity of tyrosinase enhanced the survival, immunity and competitive abilities of living cells in plants and animals [52, 60, 61, 63]. Tyrosinase is used as a potential prodrug for treatment of melanoma and other cancers where patients were successfully treated via tyrosinase activity [66].

Table 11. Viability of breast carcinoma cell lines (MCF-7) under different treatments of tyrosinase alone or combined with tyrosine.

Enzyme conc. [U/ml]	Viability [%]	
	A	B
0	100	100
6.25	78.3	67.5
12.5	77.1	62.1
25	69.2	58.3
50	64.1	55.1
100	26.4	18.8
IC_{50} (U/ml)	55.0	30.7
Viability of MCF-7 after exposure (1h) to UV was 17.9 %.		

A; MCF-7 cell line + tyrosinase (48 h). B; MCF-7 cell line + tyrosinase with tyrosine (48 h).

Conclusion

Extracellular tyrosinaseenzyme was extracted from *Saccharomyces cerevisiae,* purified, characterized and applied as medicinal agent for controlling the pathogenic microbes as well as it have antioxidant activity.

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