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Research Article

A NEW THERMOPHILIC POLYPHENOL OXIDASE FROM *Bacillus sp.*: PARTIAL PURIFICATION AND BIOCHEMICAL CHARACTERIZATION

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Abstract: Polyphenol oxidases (PPOs) catalyze the oxidation of phenolic compounds which makes them highly useful biocatalysts for various biotechnological applications. Although they are commonly found in animals, plants and fungi, recent genome analysis have shown that PPOs are also widespread in bacterial species. In this study, detection, partial purification and biochemical characterization of PPO from thermophilic *Bacillus* sp., which was isolated from a geothermal region, was achieved. The results of activity staining and activity measurements revealed the enzyme was intracellular. Partial purification was performed by acetone precipitation, ion exchange and gel filtration chromatography with 50% yield and 7.32 purification fold. Characterization studies indicated that the enzyme showed highest activity at pH 7.0 and 60 °C, was stable at temperatures between 30 and 60 °C and more than 80% of activity was retained in the pH range of 5-8. The results of effect of metal ion and other reagents on enzyme activity revealed that the enzyme was totally inhibited in the presence of DTT and sodium diethyldithiocarbamate and highly activated with copper ions. K_m and V_{max} values for the enzyme were determined as 91mM and 2.25 Δabs/min/ml, respectively.

Keywords: Polyphenol oxidase; *Bacillus* sp.; Thermophiles; Enzyme purification; Biochemical characterization

Introduction

PPOs are enzymes, belonging to a group of copper-containing metalloproteins and are members of oxidoreductases, that catalyze the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen (Queiroz *et al.*, 2008; Simsek *et al.*, 2007). In recent years PPOs have garnered significant interest because of their high capacity for oxidizing aromatic compounds. This feature makes the use of PPOs very suitable for some biotechnological applications in food industry (Simsek *et al.*, 2007; Rodriguez Couto and Toca Herrera, 2006; Polaina and MacCabe, 1974) pulp and paper industry (Rodriguez Couto and Toca Herrera, 2006), textile industry (Edwards *et al.*, 1999), medicine (Cowan *et al.*, 2000; Asanuma *et al.*, 2003; Xu *et al.*, 1998; Seo *et al.*, 2003) and environmental technology (Duran and Esposito, 2000).

PPOs are found in almost all living organisms including plants, animals, bacteria and fungi considered to be excellent sources for industrial PPO production. To date PPOs were mostly identified and studied from eukaryotic sources. Nevertheless, homology searches in protein databases and experimental data have shown that PPOs are also widespread in bacteria (Ruijssenaars and Hartmans, 2004). On the other hand little attention has been paid to bacterial PPOs and the function of the enzyme in these organisms is not fully understood. With the discovery of PPOs in bacterial species, the studies of them from these new sources have picked up in order to find more species that exhibit PPO activity (Diamantidis *et al.*, 2000; Deckert *et al.*, 1998; Claus and Decker, 2006).

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Enzymes from thermophiles with specific features have considerable potential for many industrial applications. Today, thermophilic organisms as producers of certain desired products are under interest due to their resistance to heat, denaturants solvents, proteolytic enzymes and organic solvents with respect to their counterparts from mesophiles (Fontana *et al.*, 1998). Thus, characterization of thermophiles and the stable enzymes they synthesize have been of great scientific and industrial interest for several decades.

In this paper, we have aimed to use micro-organisms that were previously isolated and characterized as a *Bacillus sp.* from Balçova Geotermal region in Izmir, Turkey (Yavuz *et al.*, 2004). Detection, partial purification and some general characterization of PPO from thermophilic *Bacillus* sp., was determined. Because until today not very much attention has been given to bacterial PPOs and the function of the enzyme in these organisms is not fully understood, our studies may be significant especially advantages of thermophilic organisms were considered in order to study of PPOs from new sources. The enzyme of interest can be used for the development of biosensors to detect phenolic compounds for various purposes, also the ability of PPO to act on phenolic compounds can be used for the degradation of phenols in industrial waste waters (Duran and Esposito, 2000).

Materials and Methods

Materials - L-DOPA was purchased from Fluka; L-tyrosine and Coomassie Brilliant Blue G-250 were purchased from Merck. Catechol, ABTS, Sephadex G-100 gel filtration resin and all chemicals for electrophoresis studies were purchased from Sigma Chem. Co.

Bacterial Strain and Growth Conditions - The bacterium, thermophilic *Bacillus* sp., was isolated from an uncontrolled thermal leak of Balçova Geothermal Region in İzmir (Yavuz *et al.*, 2004). Thermophilic *Bacillus* was cultivated overnight in yeast extract tryptone (2xYT) media, at 55 ºC and 200 rpm.

Evidence for PPO Activity in Thermophilic Bacillus sp. - Two sets of bacterial growth was carried out in a volume of 5ml 2xYT media. Following bacterial growth, discontinuous sonication was applied to bacterial cultures for 6 minutes in ice bath and the homogenate was centrifuged at 5000 rpm, 4 ºC for 20 minutes. The resulting supernatants were divided in two sets and one set was kept at 100 °C for 10 minutes. The supernatant after heat treatment was centrifuged again and supernatants of both heated or not heated samples were used as enzyme solutions in activity measurements. The activities were determined by recording the change in absorbance at 420nm with the assay mixture containing 1.5ml supernatant (heated or not heated) and 1.5ml 20mM catechol at 55 ºC. The reference cuvettes contained 1.5ml 2xYT media and 1.5ml 20mM catechol solution.

Preparation of Crude PPO Extract and Partial Purification of PPO - A 100 ml of bacterial growth culture was harvested at 5000rpm for 20 minutes. Discontinuous sonication was applied to the suspended cell pellet in 10 ml of 0.05 M sodium phosphate buffer, pH 7.0 for 6 minutes. The homogenate was centrifuged at 7500 rpm, 4 ºC for 20 minutes and the supernatant was used as crude intracellular enzyme extract.

The crude intracellular enzyme extract was subjected to total protein precipitation with two volumes of cold acetone. The resulting precipitate after an hour incubation at -20 ºC was collected by centrifugation at 15000rpm, 0 ºC for 30 minutes and the pellet was resuspended in 10.5ml 0.05M sodium phosphate buffer, pH 7.0. The enzyme solution was then loaded on to DEAE-Cellulose-SH column (Serva, Germany) and the bound proteins were eluted with a linear gradient of NaCl (0-1.0 M). The active fractions were concentrated using vacum concentrator and loaded on gel filtration (Sephadex G-100) column. Collected fractions were assayed for their protein concentrations at 280nm and for PPO activity. The active fractions were pooled and stored at -20 ºC until use for further experiments. Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method (Bradford, 1976). Bovine serum albumin was used as a standard protein.

Electrophoretic Studies and Activity Staining of PPO - Native-PAGE was performed using

acrylamide concentrations of 12% for separating and 4% for stacking gel and stained using colloidal coomassie staining solution. Specific activity staining was also performed with native polyacrylamide gel in non-denaturing conditions. The gel was equilibrated in 0.1M sodium phosphate buffer, pH 7.0, for 10 minutes at room temperature. Then the gel was transferred into a freshly prepared solution of catechol (25mM) and incubated overnight at 55 °C. The gel was washed with distilled water and stored in 5% acetic acid solution at 4°C, if needed.

Assay of PPO Activity - All the spectrophotometric assays were performed using Shimadzu UV-VIS spectrophotometer (Model 1700) with a constant temperature water circulator. The PPO activity of the samples were determined at 55 °C for 20 minutes, by recording the increase in absorbance at 420nm caused by the oxidation of catechol substrate (Simsek *et al.*, 2007). The assay mixture (1 ml in all cases) contained 10µl 2M catechol with a final concentration of 20mM, 955µl 0.1M sodium phosphate buffer at pH 7.0, and the reaction was initiated by the addition of 35µl enzymatic sample. The reference cuvettes had the same composition except for the enzyme. The enzyme activities were given as Unit or percent initial activity and Unit is defined as the amount of enzyme that causes 0.001 absorbance change in one minute.

Kinetic Analysis - Enzyme activity was measured at different concentrations of catechol varying from 5mM to 60mM, then the kinetic parameters of the enzyme, Km and Vmax, k_{cat} were determined by Lineweaver-Burk plot method.

Effect of pH on PPO Activity and Stability -The effect of pH on enzyme activity was determined under the standart assay conditions by measuring activity at different pH values ranging from 4.0 to 10.0. To determine the pH stability of the enzyme, 35µl enzyme solution was mixed with 70µl of buffer at various pHs (pH 5.0, 6.0, 7.0, 8.0, 9.0, 10.0) and then the mixture was incubated at 55°C for 1.5 hour. The residual activity was measured under standart assay conditions using 35µl of enzyme-buffer mixture.

Effect of Temperature on PPO Activity and Stability - The effect of temperature on PPO activity was examined under standart assay conditions at different temperatures ranging from 30 to 90 °C and the buffer was heated to relevant temperature before the assay. Temperature stability was assayed by incubating the enzyme solution at a range of temperatures from 30 to 80°C for 1.5 hour and then measuring the remaining activity using standart assay procedure.

Effect of Metal Ions and Various Agents on PPO Activity - The enzyme was incubated in the presence of 1mM various metal ions ($MgCl_2$, $CaCl_2$, $CuCl_2$, $MnCl_2$, LiCl, KCl, $FeCl_3$, NaCl, $ZnCl_2$) and 1 mM some agents (EDTA, sodium flouride, DMSO, DTT, sodium diethyldithiocarbamate, SDS, and Triton X-100) ion at room temperature for 10 minutes. The reaction was initiated by addition of catechol (20mM) and change in absorbance was measured under standart assay conditions.

Substrate Specificity of PPO - The substrate specificity of the enzyme was determined by measuring activity towards several monohydroxyphenol and dihydroxyphenol compounds like L-tyrosine, catechol, L-DOPA, ABTS and hydroquinone. The activities of the enzyme were measured using solutions of these compounds prepared in 0.1M sodium phosphate buffer at concentrations of 20mM for catechol and hydroquinone, 10mM for L-DOPA, 2mM for L-tyrosine and ABTS.

Results and Discussion

Existence, isolation and purification of polyphenol oxidase

The existence of PPO activity in thermophilic *Bacillus* sp. was demonstrated by two sets of bacterial growth; one set was boiled to denature the enzymes and the other was not. The results of activity measurements were given (Figure 1). It was demonstrated that the sample which was not boiled (NB) showed a significant increase in absorbance whereas boiled (B) one did not exhibited any absorbance changes. The increase in absorbance and formation of brown color in samples when assayed with catechol was due to the existence of an enzyme, not because of a compound that exists in the growth medium of

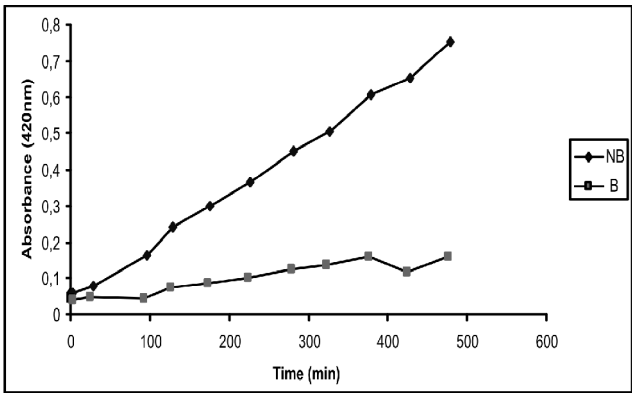

Figure 1: **Evidence for Polyphenol Oxidase Activity in Thermophilic *Bacillus* sp. by activity measurements of boiled (B) and not boiled (NB) bacterial growth**

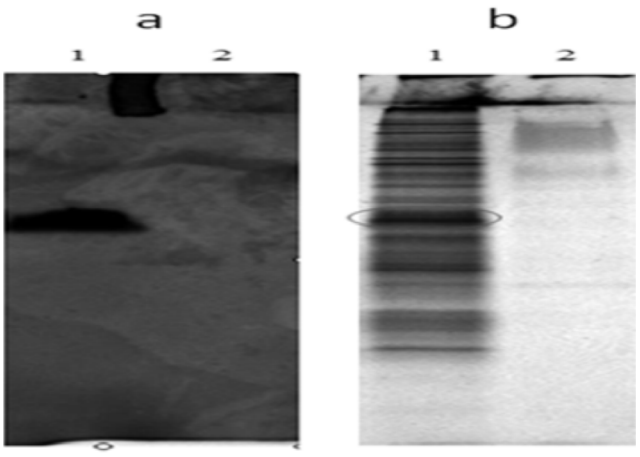

Figure 2: **Activity (a) and colloidal coomassie staining (b) of polyphenol oxidase on native polyacrylamide gels. Lane 1, supernatant of intracellular extract; lane 2, growth medium of thermophilic *Bacillus* sp.**

bacterium. In other words, the oxidation of catechol substrate was due to the existence of PPO. So it can be suggested that the responsible bacterium produces PPO enzyme that can be significant to get this enzyme from bacterial source.

Before purification of the enzyme from thermophilic *Bacillus* sp., the location of enzyme in bacterial isolate was determined with the activity measurement of both growth medium and after discontinuous sonication applied bacterial cultures. It was demonstrated that growth medium contain extracellular enzymes was poor in terms of PPO when compared with the result of intracellular extract after cell distruption. Also samples from both growth medium and intracellular enzyme extract were loaded onto native-polyacrylamide gel and subjected to activity staining with catechol solution (Figure 2). Although intracellular enzyme extract was stained with catechol, no dark band on the lane where growth medium of bacterium was loaded could be observed (Figure 2a). These results suggested that PPO from thermophilic *Bacillus* sp. was intracellular thus intracellular extract was used as starting material for purification.

An outline of the purification procedure is illustrated and the results are given in Table 1. The intracellular enzyme extract was first subjected to acetone precipitation followed by ion exchange and lasted by size exclusion chromatography with specific activity of 1757,9 U/mg, 7,32 fold purification and 50% yield. Purification fold and yield values of PPOs obtained with other bacterial species were variable up to date. First bacterial PPO from *Azospirillum lipoferum* was purified 27 fold by acetone precipitation and hydroxyapatite chromatography with 24% yield (Diamantidis *et al.*, 2000). A pH stable PPO from alkali-tolerant γ-proteobacterium JB was purified 21.5 fold by ammonium sulfate precipitation, ion exchange chromatography and preparative PAGE with 9% yield (Singh *et al.*, 2007). Better purification factor was obtained with 50 fold after purification of *Thermomicrobium roseum* PPO by ion exhcange chromatography to a final yield of 21% (Kong *et al.*, 2000). After five consecutive step by heat treatment, ammonium sulfate precipitation, ion exchange, hydroxyapatite and gel filtration chromatography for purification of *Streptomyces lavendulae* PPO, an enormous purrification factor was obtained with 261 fold with 9% purification yield (Suzuki *et al.*, 2003). By employing a one-step purification method using copper sulfate saturated ion exchange resin, PPO from *Bacillus thrungiensis* was obtained with a purification yield of 72% and high purity (Liu *et al.*, 2004).

Electrophoretic studies and activity staining

The molecular weight of partially purified enzyme was estimated to be approximately 120 kDa by comparison with the molecular weight standards (Figure 3). Although molecular weight of polyhenol oxidases from other bacterial species

vary from 120 to 14kDa (Endo *et al.*, 2003; Suzuki *et al.*, 2003; Martins *et al.*, 2002; Koschorreck *et al.*, 2008; Miyazaki, 2005; Bernan *et al.*, 1985; Lerch and Ettinger, 1972; Liu *et al.*, 2004), the molecular weight of PPO from γ-proteobacterium JB was in good agreement with our results (120kDa) (Singh *et al.*, 2007).

Native-PAGE was performed and stained with not only CBB dye to visualize all protein bands (Figure 3b) but also catechol substrate for the detection of PPO activity. The appearance of dark bands has also indicated the existence of PPO enzyme.

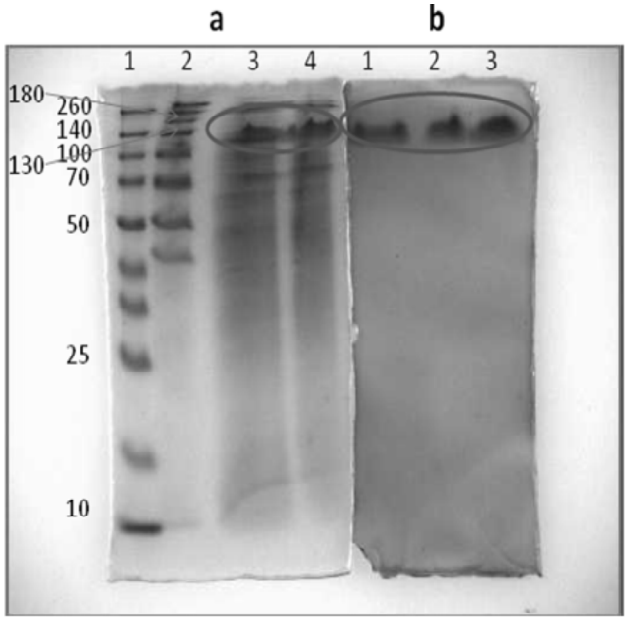

Figure 3: **Colloidal coomassie (a) and activity staining of polyphenol oxidase on native polyacrylamide gels. Lane 1 of (a) broad range protein marker (from top to bottom; 260, 140, 100, 70, 50, 40, 35, 25, 15 and 10); Lane 2 of (a) high range protein marker (from top to bottom; 300, 250, 180, 130, 100, 70, 50 and 40); Lane 3 of (a) enzyme solution after ion exchange column; Lane 4 of (a) enzyme solution after G-100 column; Lane 1 of (b) supernatant of intracellular extract; Lane 2 of (b) enzyme solution after ion exchange column; Lane 3 of (b) enzyme solution after G-100 column**

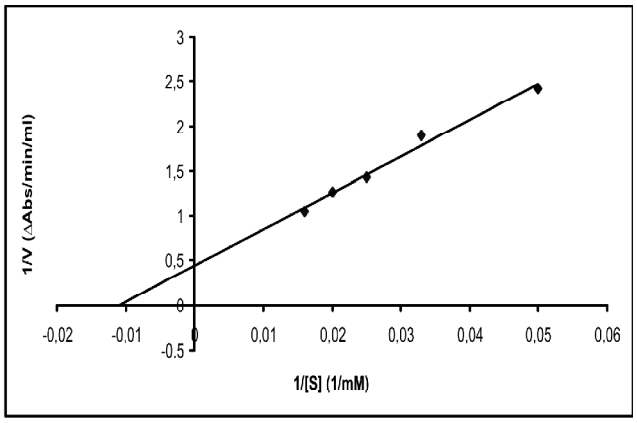

Figure 4: **Lineweaver-Burk plot of polyphenol oxidase from thermophilic *Bacillus* sp.**

Characterization of polyphenol oxidase

The PPO from thermophilic *Bacillus sp.* was found to share a number of characteristic features with other bacterial PPOs. Kinetic constants of purified thermophilic *Bacillus* PPO were obtained using catechol substrate. The Km and Vmax values of the enzyme were determined as 91mM and 2.25 Δabs/min/ml, respectively using Lineweaver-Burk plot (Figure 4). PPO from *Bacillus thuringiensis* has been reported to exhibit a Km of 34.05mM using catechol substrate (Liu *et al.*, 2004) and that from γ-proteobacterium JB, a Km value of 0.055mM using the same substrate (Singh *et al.*, 2007). In plants, PPO from apple (cv Amasya), *Ipomoea batatas* and *Amanita muscaria* have Km values of 34mM, 2.5mM and 83mM with catechol substrate , respectively (Mueller *et al.*, 1996; Oktay *et al.*, 1995). Compared to PPOs from other sources, PPO from thermophilic *Bacillus* sp. has exhibited lower affinity to its substrate, catechol.

The dependence of enzymatic activity of PPO from thermophilic *Bacillus* sp. on pH was investigated and the pH profile of the enzyme has showed a bell shaped curve with the highest activity at pH 7.0 (Figure 5). A significant loss in activity was observed upon increasing or decreasing the optimum pH value even by one pH unit. The enzyme exhibited low activity at pH 5.0 and no activity at pH 4.0. On the other hand at alkaline pH values, the enzyme is not effected much as in acidic conditions and showed 30% of its activity. Similar to thermophilic *Bacillus* sp. PPO, optimum pH value close to neutrality have been reported for PPOs from other bacterial species such as; *Streptomyces michiganensis* (pH 7.0) (Philipp *et al.*, 1991), *Pseudomonas putida* (pH 7.0) (McMahon *et al.*, 2007), *Vibrio tyrosinaticus* (pH 6.6-7) (Pomerant and Murthy, 1974), *Streptomyces glaucescens* (pH 6.8) (Lerch and Ettinger, 1972), *Streptomyces griseus* (Endo *et al.*, 2003) and γ-proteobacterium JB (pH 6.5) (Bains *et al.*, 2003). Nevertheless, acidic and alkaline optimum pH values for PPOs from bacterial species such as *Thermomicrobium roseum* (pH 9.5)

Table 1
Purification of polyphenol oxidase from thermophilic *Bacillus* sp.

Purification Step	*Volume (ml)*	*Activity (U/ml)*	*Total Activity (U)*	*Protein (mg/ml)*	*Total protein (mg)*	*Specific Activity (U/mg)*	*Yield (%)*	*Purification (Fold)*
Cell Extract	10	496	4956	2,07	20,66	239,93	100	1
Precipitation	10.5	382	4015	1,33	13,99	286,95	81	1,19
Ion Exchange	8	368	2944	0,44	3,53	833,71	59,4	3,47
Gel Filtration	7	355	2482	0,20	1,41	1757,9	50	7,32

(Kong *et al.*, 2000), *Bacillus thuringiensis* (pH 9.0) (Liu *et al.*, 2004) and *Bacillus licheniformis* (pH 4.2) (Koschorreck *et al.*, 2008) have also been observed.

The pH stability of the enzyme was examined by incubating the enzyme in various buffers for 1.5 hour. The residual activities were measured under standart assay conditions and the activity of enzyme which was not subjected to pH treatment for 1.5 hour was regarded as hundred percent (Figure 6). The PPO enzyme retained more than 80% of its activity in the pH range of 5-8, however lost 40% of its activity at pH 9. The enzyme was quite stable compared to PPOs from other bacterial sources as it retained most of its activity through a broad range of pH after 1.5 hour incubation period. It was reported that *Thermomicrobium roseum* PPO retained more than 70% activity in the pH range of 8.5-10.0 but lost approximately 75% of activity below pH 6.0 and above 11.0 upon incubation in various buffers at 4 °C for 20 hours (Kong *et al.*, 2000). Another PPO from *Pseudomonas putida* was incubated in various buffers for 30 minutes and retained 99% and 80% of activity acroos a broad range of pH values (pH 4-7 for monophenolase and pH 4-9 for diphenolase) (McMahon *et al.*, 2007).

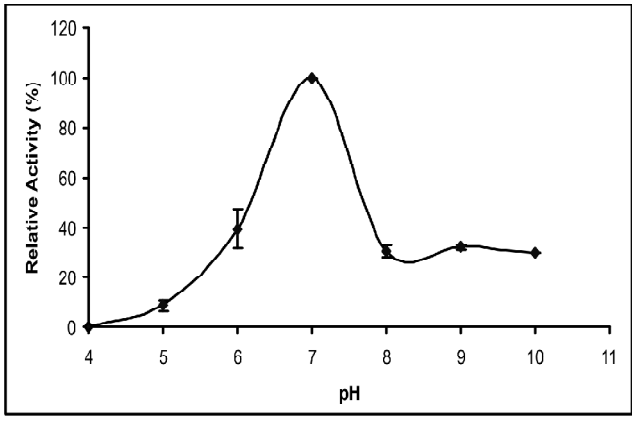

Figure 5: **Effect of pH on polyphenol oxidase activity**

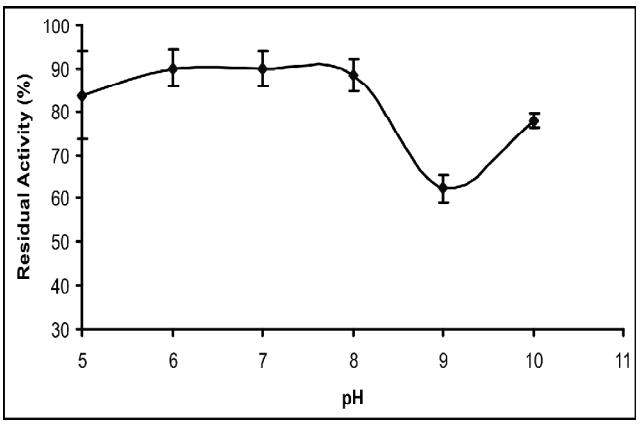

Figure 6: **pH stability of polyphenol oxidase**

PPO activities at different temperatures ranging from 30 to 90 °C were measured (Figure 7). The results of these measurements indicated that the enzyme showed highest activity at 60°C. The activity of the enzyme was stimulated upon heating up to 60 and 70 °C. However, at temperatures above 70 °C, a decrease in PPO activity was observed with 82% and 35% of the activity at 80 and 90 °C, respectively. Such a high temperature or even higher temperatures of maximal activity were also observed for PPOs obtained from other bacteria. The temperature maxima of 92 °C was recorded with *Thermus thermophilus* PPO (Miyazaki, 2005), 85 °C with *Bacillus licheniformis* PPO (Koschorreck *et al.*, 2008), 75 °C with *Bacillus thuringiensis* (Liu *et al.*, 2004) and CotA protein of *Bacillus subtilis* (Martins *et al.*, 2002), 70 °C with *Thermomicrobium roseum* (Kong *et al.*, 2000), and 55 °C with both *Bacillus* sp. HR03 (Dalfard *et al.*, 2006) and γ-proteobacterium JB (Bains *et al.*, 2003).

The thermal stability profile of PPO was created by measuring enzyme activity after 1.5 hour incubation at different temperatures (Figure 8). These results showed that the enzyme

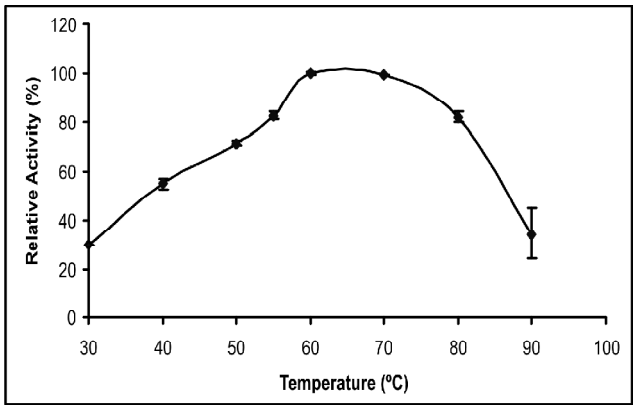

Figure 7: **Effect of temperature on polyphenol oxidase activity**

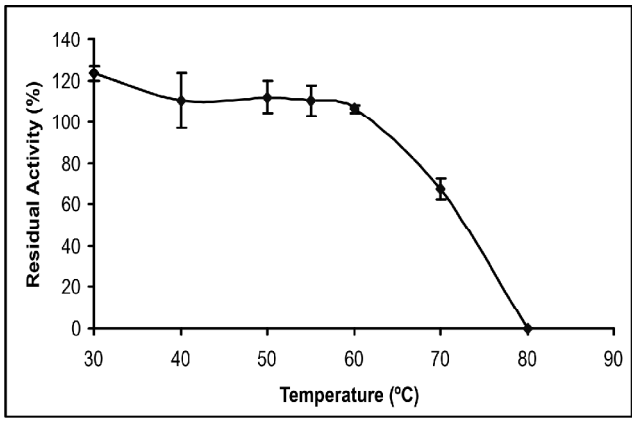

Figure 8: **Thermal stability of polyphenol oxidase**

was fairly stable for 1.5 hour at temperatures up to 60 °C. At temperatures above 60 °C, a decline in activity was observed. Although the enzyme retained nearly 70% of its activity at 70 °C, the activity was completely lost upon incubation for 1.5 hour at 80 °C. Thermal stability of polyphenol oxidase from *Bacillus* sp. was compared with other thermophilic polyphenol oxidases (Table 2). It was reported that a hyperthermophilic PPO from *Thermus thermophilus* was found to be resistant to incubation at 85 °C for 10 minutes, also the enzyme retained two-thirds of its activity at 100 °C for 10 minutes (Miyazaki, 2005). Another PPO from *Bacillus thuringiensis* was most stable at 75 °C (Liu *et al.*, 2004). *Thermomicrobium roseum* PPO was very stable between 30-70 °C with 10 minutes incubation period (Kong *et al.*, 2000). On the other hand *Streptomyces* PPO had a half-life of 1-5 minutes at 60 °C (Huber and Lerch, 1988).

The effect of various metal ions on PPO activity was obtained in Figure 9. In the presence of Zn^{2+} and K^{+}, PPO activity was not effected but an increase in enzyme activity was observed in the presence of Ca^{2+}, Cu^{2+} and Mg^{2+}. Cu^{2+} caused a significant amount of activation on PPO activity. This outcome is not surprising since PPOs are copper containing enzymes and copper is essential for catalytic activity. Similar activator effect of copper on the activity of PPO from *Thermomicrobium roseum* (Kong *et al.*, 2000) and *Bacillus thuringiensis* (Liu *et al.*, 2004) were also reported. Also addition of copper to the growth medium of *Bacillus* (HR03) was found to increase the melanin production (Dalfard *et al.*, 2006).

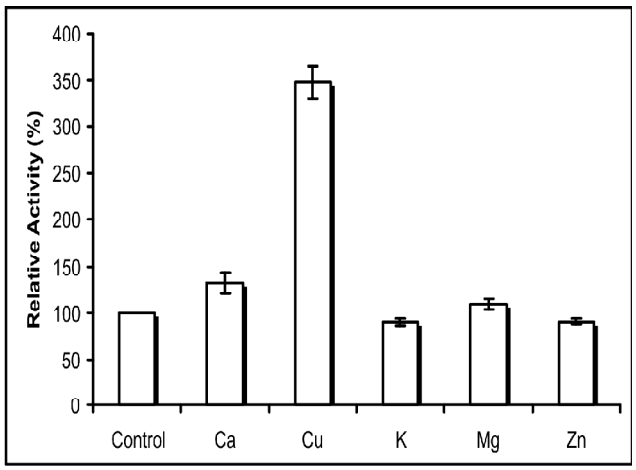

Figure 9: **Effect of metal ions on polyphenol oxidase activity**

Table 2
Thermal stability comparison of polyphenol oxidase from *Bacillus* sp. with other thermophilic polyphenol oxidases

PPO Source	*Incubation Temperature (°C)*	*Incubation Period (min)*	*Reference*
Bacillus sp.	60	90	-
Thermus thermophilus	85	10	Miyazaki, 2005
Bacillus thuringiensis	75	10	Liu *et al.*, 2004
Thermomicrobium roseum	70	10	Kong *et al.*, 2000
Streptomyces	60	5	Huber and Lerch, 1988

In addition to metal ions, several agents were also tested for the action of PPO (Table 3). It was indicated that DTT and sodium diethyldithiocarbamate are strong inhibitors for PPO from thermophilic *Bacillus* sp. Even in the presence of 1mM of these agents, the enzyme exhibited no activity under standart assay conditions. Sodium diethyldithiocarbamate is a sulfur containing compound and used as a chelating agent for transition metal ions. This agent is known as potent inhibitor of tyrosinase activity of PPOs and it was suggested that this compound may cause inhibition by forming complexes with copper atoms in the active site (Kong *et al.*, 2000). Sodium fluoride, which is regarded as a typical inhibitor for laccase activity of PPOs, did not exhibit a strong inhibitory action on PPO in this study. Sodium fluoride with a concentration of 5mM inhibited the PPO activity of *Bacillus thuringiensis* (Liu *et al.*, 2004). Thus, higher concentrations of this agent may be required for the inhibiton of thermophilic *Bacillus* PPO. DMSO and some detergents such as SDS and Triton X-100 did not cause much effect on activity such that the enzyme showed approximately 97% of its activity in the presence of those detergents and 91% in the presence of DMSO. The effect of a chelating agent, EDTA, on enzyme activity was also investigated. Since the active site of PPO contains copper ions and they are involved in catalytic activity, chelating compounds would inhibit PPO activity by removing copper ions. Interestingly, the presence of 1mM EDTA barely effected the action of PPO and the enzyme showed 95% of its activity. However PPOs from *Streptomyces griseus* and *Bacillus thuringiensis* showed 67% and 72% of their activity in the presence of EDTA with same concentration, respectively (Liu *et al.*, 2004; Endo *et al.*, 2003). In contrast to inhibitory effect, the activator effect of EDTA on *Bacillus thuringiensis* PPO, in the concentration range of 200-400mM, have been reported (Liu *et al.*, 2004).

The substrate specificity of PPO from thermophilic *Bacillus* sp. was determined by measuring enzyme activity using catechol, hydroquinone, L-tyrosine, ABTS and L-DOPA at appropriate wavelengts (Table 4). Catechol has demonstrated the best substrate for PPO in terms of activity compared to other substrates used here.

Table 3
Effect of various agents on polyphenol oxidase activity

Agent	*Concentration*	*Relative activity (%)*
Control	-	100
Sodium diethyldithiocarbamate	1mM	0
Sodium fluoride	1mM	89
DTT	1mM	0
EDTA	1mM	95
SDS	1mM	97
Triton X-100	5%	98
DMSO	1mM	91

Table 4
Substrate specificity of polyphenol oxidase

Substrate	*Wavelength (nm)*	*Relative Activity (%)*
ABTS	420	2,24
Catechol	420	100
L-DOPA	475	20
L-tyrosine	475	0,78
Hydroquinone	420	8,20

Conclusion

Although PPOs are widespread in many organisms including bacteria, bacteria originated PPO identified in thermophiles are very rare. It is of interest to provide a PPO produced by thermophilic bacteria, a novel enzyme source for use in the oxidation of colored substances and oxidation of polyphenol-containing substances and also for use in cleaning and may have great potential in industrial applications. According to results of our research, the PPO can be produced highly efficiently from the bacterial sources in an economical way, especially thermophilic ones.

Abbreviations

PPO, polyphenol oxidase; DTT, dichlorodiphenyltrichloroethane; L-DOPA, L-3,4-dihydroxyphenylalanine; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); 2xYT, yeast extract tryptone; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

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