Production of a novel bifunctional catalase-phenol oxidase of *Scytalidium thermophilum* in the presence of phenolic compounds

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Abstract: A novel bifunctional catalase with additional phenol oxidase activity (CATPO) is produced by the thermophilic fungus, *Scytalidium thermophilum*, in a growth-associated manner. In order to study the biological significance of this dual enzyme in relation to phenolic compounds, 14 phenolics were tested for their effect on growth and CATPO production. It was determined that some phenolics exerted a negative effect on growth (catechol, coumaric acid, hydroquinone, kaempferol, myricetin), while others either did not influence growth in a negative manner or enhanced growth by a maximum of 50% increase in biomass (caffeic acid, chlorogenic acid, catechin, gallic acid, resorcinol, vanillic acid). Hydroquinone and myricetin showed an antifungal effect and enhanced CATPO production, while catechol, coumaric acid, and kaempferol decreased CATPO production and showed a toxic effect at higher doses. In general, phenolics acting in an antioxidant manner exhibited a reverse interrelation with CATPO production. These results suggest that the presence of antioxidant phenolic compounds has an effect on enhancing its antioxidant activity; enzyme is therefore no longer required.

Key words: Antioxidant, catalase, phenol oxidase, phenolic compound, *Scytalidium thermophilum*, thermophilic fungus

*Scytalidium thermophilum’un özgün çift aktiviteli katalaz-fenol oksidazının fenolik maddeler varlığında üretimi*


Anahtar sözcükler: Antioksidan, katalaz, fenol oksidaz, fenolik madde, *Scytalidium thermophilum*, termofilik mantar
Introduction

Catalases (EC 1.11.1.6), belonging to the oxidoreductase family, are a group of metalloenzymes with the ability to catalyze the decomposition of hydrogen peroxide into water and dioxygen (1). Four classes of catalases have been defined, including monofunctional heme-containing catalases, catalase-peroxidases, manganese catalases, and minor catalases (2,3). Although catalases have been studied for many years, a secondary peroxide-independent oxidative function of catalase is regarded as a new discovery in the literature. The first bifunctional catalase-oxidase was introduced by Vetrano et al. (4) for a mammalian catalase, where the authors state that the enzyme oxidizes most of the functionally important phenolic compounds, suggesting a significant biological role for this dual activity. The first bifunctional catalase-oxidase, characterized in detail in the literature, is the catalase-phenol oxidase (CATPO) of the thermophilic fungus Scytalidium thermophilum (5). CATPO is among the monofunctional heme-containing catalases and shows phenol oxidase activity resembling catechol oxidases as well as having some features of laccases (6).

Both catechol oxidases (E.C. 1.10.3.1) and laccases (E.C. 1.10.3.2) are copper-containing enzymes responsible for the oxidation of phenols into quinones (7). There are a number of reports available that show that phenol oxidases may have additional catalase activity (8-11), suggesting that the dual existence of catalase and phenol oxidase activities is more widespread in nature and may have important implications as to the antioxidant mechanism of biological systems. This is due to the fact that catalases cope with reactive oxygen species (ROS) generated as a result of oxidative stress and many phenolic compounds are known to be effective antioxidants, although they may also function as pro-oxidants in a dose-dependent manner (12).

The purpose of the current study was to study the biological significance of the phenol oxidase activity of the dual catalase (CATPO) from S. thermophilum in relation to functional phenolic compounds. In order to do this, 14 phenolic compounds were selected and tested for their effect on the growth of S. thermophilum and CATPO production in order to establish the relationship of dose-dependent influence on growth and CATPO production.

Materials and methods

Microbial strain and maintenance

S. thermophilum (type culture Humicola insolens, ATCC No. 16454) kindly provided by ORBA, Istanbul, was inoculated onto YpSs agar plates (13) and incubated at 45 °C for 4-5 days until sporulation was complete. These agar plates can be stored at 20 °C for up to 2 months. Spores from these stock cultures were inoculated into a liquid pre-culture media (5 mL) known as YpSs broth, which contains 1% glucose instead of starch as a carbon source. After 24 h of incubation at 45 °C, the pre-culture (5 mL) was transferred into the main culture (250 mL) supplemented with copper sulfate. Cultures were incubated in a shaker incubator at 45 °C with a shaking rate of 155 rpm.

Cultivation of S. thermophilum and biomass determination

For the purposes of our study, 14 phenolic compounds were chosen according to their different chemical structures. Each of the phenolics was added to the growth medium of S. thermophilum at various concentrations. Preliminary experiments with catechol were performed to determine the concentration range of phenolic compounds, which exhibited a strong inhibition of fungal growth at concentrations above 2 mM and induction around 0.5 mM. Considering this, simple diphenols were used, including catechol, hydroquinone, and resorcinol, as were phenolic acids like gallic acid, vanillic acid, caffeic acid, chlorogenic acid, and coumaric acid at concentration levels of 0.1, 0.3, 0.5, 1, and 2 mM. For flavonoids, a lower and narrower concentration range was chosen (14). Flavonols kaempferol, quercetin, and myricetin were added at 0.0025, 0.03, 0.05, 0.1 and 0.2 mM; the levels were set at 0.04, 0.08, and 0.16 mM for catechin and 0.01, 0.02, and 0.04 mM for epicatechin. The disparity in the concentrations used was due to the flavonoids’ various levels of solubility in the culture medium used. Resveratrol, belonging to stilbene family, was used in a concentration range between 0.1 and 1 mM at 0.2 mM intervals.

Culture supernatant was removed by filtration through Whatman® No.1 filter paper. Separated cell biomass was dried overnight at 60 °C for the determination of fungal biomass.
Enzyme assays

Catalase and phenol oxidase assays were determined spectrophotometrically using a temperature-controlled spectrophotometer (Shimadzu UV-1700). Each individual assay was performed in triplicate.

Catalase activity was measured at 60 °C in 100 mM sodium phosphate buffer (pH 7) using 10 mM H₂O₂ as substrate (5). The decrease in absorbance at 240 nm was monitored. Enzyme activity was determined using the initial rate of the reaction and the extinction coefficient for H₂O₂ was taken as 39.4 M⁻¹ cm⁻¹ (15). One enzyme unit was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol H₂O₂ per min.

Phenol oxidase activity was determined by monitoring the increase in absorbance at 420 nm. The reaction mixture consisted of 0.5 mL of 100 mM catechol solution in 100 mM phosphate buffer (pH 7) as substrate, 0.5 mL of enzyme solution at different dilution rates, and 1 mL of 100 mM phosphate buffer at 60 °C (5,6). Enzyme activity was determined using the initial rate of the reaction and the extinction coefficient as 3450 M⁻¹ cm⁻¹ for catechol (6) and one enzyme unit was defined as the amount of enzyme required for the formation of one nanomole of product per minute.

Statistical analysis

One-way analysis of variance (ANOVA) was used to test the effect of phenolic compounds on fungal growth and CATPO production. All statistical analyses were performed with MINITAB 13. The confidence level of this study was established as 95%.

Results and discussion

The effect of phenolic compounds on growth and CATPO production

A total of 14 different phenolic compounds were added to the growth medium of Scytalidium thermophilum at different concentrations and biomass and catalase-phenol oxidase (CATPO) production were analyzed on the fifth day of growth. Extracellular catalase and phenol oxidase activities were used as indicators of CATPO production.

Depending on the results obtained, S. thermophilum exhibited different sensitivity towards different phenolic compounds. With the exception of quercetin and epicatechin, all of the phenolic compounds tested were found to have a significant effect on either growth and/or catalase and phenol oxidase production (Table).

Table. The dose-dependent effects of phenolic compounds on S. thermophilum CATPO production and biomass generation (Statistically significant values are indicated in bold).

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>% Relative max biomass (g/L)</th>
<th>% Relative max CATPO production (U/g)</th>
<th>% Relative min biomass (g/L)</th>
<th>% Relative min CATPO production (U/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no phenol)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Catechol (0.1 mM)</td>
<td>119</td>
<td>121</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Hydroquinone (0.3 mM)</td>
<td>92</td>
<td>131</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Coumaric acid (0.1 mM)</td>
<td>110</td>
<td>107</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>Kaempferol (0.0025 mM)</td>
<td>102</td>
<td>115</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>Myricetin (0.03 mM)</td>
<td>94</td>
<td>149</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Resorcinol (0.1 mM)</td>
<td>141</td>
<td>56</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Gallic acid (0.3 mM)</td>
<td>126</td>
<td>128</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Vanillic acid (0.3 mM)</td>
<td>128</td>
<td>71</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid (0.1 mM)</td>
<td>124</td>
<td>147</td>
<td>107</td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid (0.3 mM)</td>
<td>141</td>
<td>101</td>
<td>122</td>
<td></td>
</tr>
<tr>
<td>Catechin (0.04 mM)</td>
<td>136</td>
<td>90</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Quercetin (0.03 mM)</td>
<td>105</td>
<td>126</td>
<td>108</td>
<td></td>
</tr>
<tr>
<td>Epicatechin (0.02 mM)</td>
<td>115</td>
<td>93</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Resveratrol (0.3 mM)</td>
<td>112</td>
<td>111</td>
<td>236</td>
<td></td>
</tr>
</tbody>
</table>
Production of a novel bifunctional catalase-phenol oxidase of *Scytalidium thermophilum* in the presence of phenolic compounds

In general, 2 different behaviors of phenolic compounds were observed on the growth of *S. thermophilum*. One group of phenolics presented an antifungal/toxic effect and thus had a negative influence on growth in a dose-dependent manner. Another group of phenolic compounds had an overall significantly positive effect on growth in a dose-dependent manner.

Among the first group of phenolics, catechol, coumaric acid, and kaempferol were found to be less effective at lower doses and exerted their effects at higher doses (Figure 1a). These compounds were said to exert a toxic effect only at higher doses. On the other hand, hydroquinone and myricetin caused a steady decrease in growth with respect to biomass and, thus, were said to exert an antifungal effect (Figure 1b).

The second, growth-promoting group of phenolics, namely resorcinol, gallic acid, caffeic acid, catechin, vanillic acid, and chlorogenic acid, were more effective at lower doses (Figure 2); at higher doses, growth was again decreased. Growth suppression was not observed with respect to the control (Figure 2).

![Figure 1](image1.png)

**Figure 1.** Different trends of growth inhibition observed when *S. thermophilum* was grown on catechol (0.1-2 mM), coumaric acid (0.1-2 mM), and kaempferol (0.0025-0.12 mM) (a) versus growth on hydroquinone (0.1-2 mM) and myricetin (0.0025-0.2 mM) (b). Growth was at 45 °C at 155 rpm in medium containing glucose as the carbon source. The concentration ranges for kaempferol and myricetin are presented in the top X-axis of graph (a) and (b).

![Figure 2](image2.png)

**Figure 2.** Effect of resorcinol (0.1-2 mM), gallic acid (0.1-2 mM), caffeic acid (0.1-2 mM), catechin (0.04-0.16 mM), vanillic acid (0.1-2 mM), and chlorogenic acid (0.1-2 mM) on the growth of *S. thermophilum* at 45 °C, 155 rpm, and in medium containing glucose as the carbon source (see Materials and methods). The concentration range for catechin is presented in the top X-axis.
Figure 3 shows *S. thermophilum* extracellular catalase and phenol oxidase activities. In accordance with our findings, CATPO production is constitutive and simultaneous to microbial growth in a glucose-containing medium, indicating growth-associated production of the enzyme. Under the stated conditions, CATPO is the only secreted phenol oxidase in the medium (6).

In the presence of phenolic compounds, positive or negative effects on CATPO production could be observed irrespective of the changes in growth. The 2 general trends observed, namely enhancement and suppression of CATPO production, are shown in Figure 4.

When different phenolics are compared with respect to their positive and negative effects on CATPO production, the most drastic suppression in enzyme production was observed in resorcinol (95% decrease, P < 0.001), followed by catechol (62% decrease, P < 0.001) and vanillic acid (43% decrease, P < 0.01). The highest level of enhancement in CATPO production was observed with myricetin (49% increase, P < 0.05), followed by caffeic acid (47% increase, P < 0.05) and resveratrol (11% increase, P < 0.05). Myricetin showed a positive effect even at the highest concentrations tested. Interestingly, resveratrol displayed a specific inductive effect on phenol oxidase activity, which was approximately 2.4 times higher than the control. As stated above, CATPO appears to be the only phenol oxidase secreted; however, the possibility of another phenol oxidase being secreted in the presence of resveratrol could not be excluded. Therefore, the effect of resveratrol on the phenol oxidase activity of purified CATPO was also analyzed. The same activity enhancement was observed, indicating that the triggering effect is directly on CATPO (data not shown).

Among the eukaryotes, thermophilic fungi are the most heat-resistant organisms. It is suggested that they have evolved from their mesophilic counterparts by adaptation to higher temperatures (16). It is proposed that they continuously produce antioxidant chemicals and enzymes to compensate for the excessive generation of reactive oxygen species due to heat-stress (16). *S. thermophilum* is an important thermophilic fungus of mushroom compost and it is known to trigger the production of *Agaricus bisporus* mushrooms (17). The bifunctional CATPO (catalase-phenol oxidase) of *S. thermophilum* is constitutively produced in glucose-containing medium, in a growth-associated manner. This supports the suggestion of Feofi-lova and Tereshina (16) that antioxidant enzymes and chemicals are continuously produced.

An additional phenol oxidase activity of catalases appears to be more widespread in nature than expected (5), a finding which may have implications for the general interrelationship of dual catalases and the antioxidant mechanism. Thus, *S. thermophilum* may act as a model system for the study of bifunctional catalase-phenol oxidases.

As a first step in the study of the biological significance of CATPO from *S. thermophilum*, the
present study aimed to establish the interrelationship between phenolic compounds and the growth and CATPO production of *S. thermophilum*. With this goal in mind, 14 phenolic compounds were selected to provide a wide range of different chemical structures of phenolics that could be analyzed. Analyses were performed on both the fourth and fifth days of growth. The same trends were observed on both days, however, and therefore only the fifth day results are presented here.

In general, most phenolic compounds showed a significant effect on both growth and CATPO production. Other than those showing no significant difference, the effect on growth was classified as those exerting a positive or negative overall effect. Those exerting a negative effect were further classified into those showing antifungal effect (hydroquinone and myricetin) and those showing a toxic effect at higher doses (catechol, coumaric acid, and kaempferol). This sub-category was generated because, as the...
concentration of hydroquinone and myricetin increased, biomass decreased in a continuous manner. However, those compounds suggested to exert a toxic effect generally showed an initial increase in growth prior to a relative decrease at higher doses.

The toxic effect of some of these phenolic compounds appears to be related to the site and number of hydroxyl groups on the phenolic compound. Among diphenols, resorcinol is an $m$-diphenol and the only difference between this compound and catechol ($o$-diphenol) and hydroquinone ($p$-diphenol) is the position of the second hydroxyl group on the molecule. Catechol and hydroquinone have been found to be significantly more toxic than resorcinol, possibly due to the fact that resorcinol is less oxidized under the conditions existing in the culture medium and therefore does not produce sufficient levels of oxygen radicals (18,19). In addition, the flavonoid myricetin possesses 3 hydroxyl groups in the B-ring and its antifungal activity is thought to be due to its multiple hydroxyl groups. The increased production of hydroxyl radicals in a Fenton system resulting from the multiple hydroxyl groups in B-ring of flavonoids was reported by Hanasaki et al. (20).

In general, the growth stimulatory compounds (resorcinol, gallic acid, caffeic acid, catechin, vanillic acid, and chlorogenic acid) either suppressed CATPO production in a dose-dependent manner or had no effect. Such a trend is likely to occur due to the antioxidant activity of these compounds. Indeed, resorcinol has been found by the Trolox Equivalent Antioxidant Capacity (TEAC) assay to have higher antioxidant activity than the structurally related compounds, catechol and hydroquinone (21). It is suggested by the authors that this antioxidant activity results from the antioxidant capacity of resorcinol and its oxidation product(s) (21,22). Flavonoids and phenolic acids, having an ortho-diphenolic nature, are known as strong antioxidants (23). This structure is important for acting as free radical scavengers and metal chelators due to the additional resonance stabilization and $o$-quinone formation (12,24-27). Thus, when strong antioxidants are present in the medium to support the antioxidant system of the thermophilic $S.\ thermophilum$, CATPO and other antioxidant enzymes and chemicals may be less required to cope with reactive oxygen species (ROS) likely to be generated at the high growth temperatures of the fungus (16).

In the presence of growth inhibitory compounds, it was observed that CATPO production was also negatively influenced. This is likely due to the functional loss of the healthy cells, thereby preventing cells from effectively producing essential enzymes. In contrast, phenolics exerting an antifungal activity enhanced CATPO production in a dose-dependent manner, suggesting a role for CATPO in the defense mechanism of $S.\ thermophilum$. Although catalases are not considered to be defense enzymes, phenol oxidases are shown to have roles in antifungal defense (28). Thus CATPO, which bears the characteristic features of catalases, is also capable of acting as a typical phenol oxidase.

Another unexpected and interesting observation was the phenol oxidase-specific, activity-triggering effect of resveratrol, which is known to be a compound enhancing the life-span of yeast and mammals (29-31). These findings deserve further investigations and are in progress in our laboratory. Of particular interest are the chemical structures of the oxidation products and their antioxidant capacities with respect to their un-oxidized forms.

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