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Synthesis of Antioxidant Propyl Gallate Using Tannase from *Aspergillus niger* van Teighem in Nonaqueous Media

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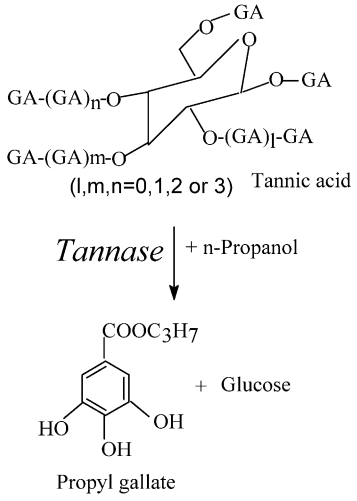
Abstract—Tannase from *Aspergillus niger* van Teighem has been used for synthesis of food additive antioxidant propyl gallate by direct transesterification of tannic acid. The optimized yield of 86% was obtained by using simultaneously pH tuned enzyme, immobilized on Celite and using the right amount of water in the non aqueous media.

Propyl gallate is frequently used as a food additive since it acts as an antioxidant. Weetal[1] had explored the possibility of its preparation by esterification of gallic acid by tannase. Actually, gallic acid is costlier than propyl gallate.[2] The alternative route suggested by Gaathon et al.[3] of tannase catalysed transesterification of tannin makes much better sense. They have used a reverse micellar system and reported a yield of 51%.

In recent years, there have been considerable developments in the use of enzymes in low water systems for organic synthesis.[4] Recently, a tannase from *Aspergillus niger* van Teighem and its applications have been reported.[5] In this work, we describe the use of this tannase for the synthesis of propyl gallate by direct transesterification of tannic acid (Sigma Chemical Company, USA) using propanol itself as the organic reaction media under low water conditions. The product was monitored by HPLC[6] and the percentage product formed was calculated from the standard curve for propyl gallate (Sigma Chemical Company, USA).

Tannase preparations are known to display two activities.[7] The first one is an esterase activity of which gallic acid esterase activity has been widely reported. The second one is called depsidase activity which hydrolyzes esters of m-digallic acids. Thus, tannase under low water conditions can carry out the following reaction (Scheme 1).

The phenomenon of 'pH memory' is now fairly well established in nonaqueous enzymology.[8] Tannase used in the present work has been shown to have two pH optima of pH 5.0 and 6.0.[5b] It was found that pH tuning at pH 6.0 gave better conversion (Table 1). Further work was carried out by enzyme tuned at pH 6.0. Nonpolar media are known to give better enzyme activities as compared to polar media. However, any effort to use a common solvent with less polarity did not work out since the substrate tannic acid was found to have less than adequate solubility.



Scheme 1. Transesterification of tannic acid to propyl gallate in the presence of *n*-propanol using tannase.

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Table 1. Optimization of the reaction media for propyl gallate synthesis

Enzyme	Product yield (%)
Untuned[a] Reaction medium: *n*-propanol	0.2
Tuned** tannase in 0.05 M citrate buffer, pH 5.0;[b] reaction medium: *n*-propanol	6.7
Tuned tannase in 0.05 M citrate buffer, pH 6.0;[c] reaction medium: *n*-propanol	11.5
Tuned tannase in 0.05 M citrate buffer, pH 6.0 and 98% (w w^{-1}) KCl was also added during lyophilization;[d] reaction medium: *n*-propanol	0.2
Tuned tannase in 0.05 M citrate buffer, pH 6.0 and ultrasonicated;[e] reaction medium: *n*-propanol	3.6

*Untuned: The enzyme is lyophilized in distilled water i.e., pH of the enzyme solution is not adjusted before lyophilization.

**Tuned: Adjusting pH of the enzyme solution by dissolving the enzyme powder in respective buffers (desired pH value) before lyophilization.

The experiments were carried out in duplicates and the error was within ± 5%.

[a]Tannase powder (50mg) was added to 1.0 mL of 10 mM tannic acid in *n*-propanol which is the reaction media.

[b]Tannase (50 mg) tuned in 50 mM citrate buffer, pH 5.0 was added to the reaction media (1.0 mL of 10 mM tannic acid in *n*-propanol).

[c]Tannase (50 mg) tuned in 50 mM citrate buffer, pH 6.0 was added to the reaction media (1.0 mL of 10 mM tannic acid in *n*-propanol).

[d]Tannase (50 mg) tuned in 50 mM citrate buffer, pH 6.0 and 98% KCl (w w^{-1})[4d] was added to the reaction media (1.0 mL of 10 mM tannic acid in *n*-propanol).

[e]Tuned tannase (50 mg in 50 mM citrate buffer, pH 6.0) was given a burst in ultrasonicator for 30 s and then added to 1.0 mL of 10 mM tannic acid in *n*-propanol.

In recent years, the presence of KCl has been shown to dramatically enhance the activity of some hydrolases in nearly anhydrous organic media.[4] In the case of tannase, the presence of KCl actually decreased the product yield to a negligible level. Ultrasonication of enzyme powder is another approach which has sometime improved their performance under low water conditions.[4] Again, this approach actually decreased the product yield in this case.

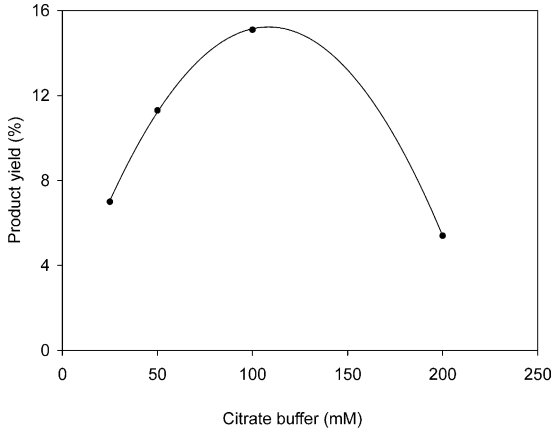

Figure 1. Effect of molarity of the tuning buffer on the ester synthesis. Tannase (5 mg) was lyophilized in citrate buffer of varied molarity (25, 50, 100, 200 mM), pH 6.0. The separately lyophilized enzyme powders were added to reaction media containing 10 mM tannic acid in *n*-propanol.

Triantafyllou et al.[9] have shown that in the case of α-chymotrypsin and lipase, one can improve the rate of reaction by optimizing the molarity of the tuning buffer. Figure 1 shows that the product yield could be marginally improved to 15% by using 100 mM citrate buffer for tuning the enzyme at pH 6.0 instead of 50 mM.

The above experiments were done using 50 mg solid enzyme. The reaction was repeated with 1, 2, 5, 25 and 50 mg enzyme powders lyophilized in 100 mM citrate buffer, pH 6.0. While, no significant product could be detected with 2 mg or less enzyme powders, 5 mg of enzyme powder were found to be optimum with 32% product yield. The decrease in yield with higher amounts of enzyme was presumably due to the highly viscous nature of the reaction media (data not shown). Therefore, 5 mg enzyme of enzyme were used for further experiments.

Addition of a limited amount of water to such media is known to increase reaction rates. This is believed to be due to improvement in enzyme flexibility in such media.[9] Varying amounts of water viz., 0.5, 1.0, 1.5, 2.0% were added to the reaction mixture consisting of low water *n*-propanol, tannic acid (10 mM) and the lyophilised powder. Figure 2 shows that addition of 1% water gave the optimum product yield of 68.4%. Addition of sorbitol has been reported to improve enzyme rates in low water media.[9] Sugars like sorbitol are presumed to act similar to the essential water layer by disrupting intramolecular hydrogen bonding on the enzyme surface. Figure 3 shows that sorbitol indeed helps but is not as effective as water for imparting necessary flexibility by substituting intramolecular hydrogen bonding with hydrogen bonding with the additive.

Finally, immobilization has been suggested as a way to increase surface area as well as stability of enzyme in nonaqueous media.[10] Celite has been frequently used as an economical matrix. It was found that tannase immobilized on Celite-545 gave 72% product yield.[11] Further, the propyl gallate synthesis increased to 86%

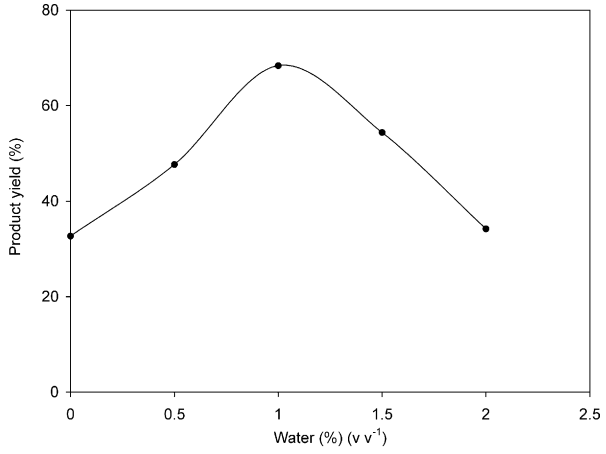

Figure 2. Effect of addition of water to the reaction media. To the tuned tannase (5 mg in 100 mM citrate buffer, pH 6.0) varied amounts of water viz., 0.5, 1.0, 1.5, 2.0% were added.

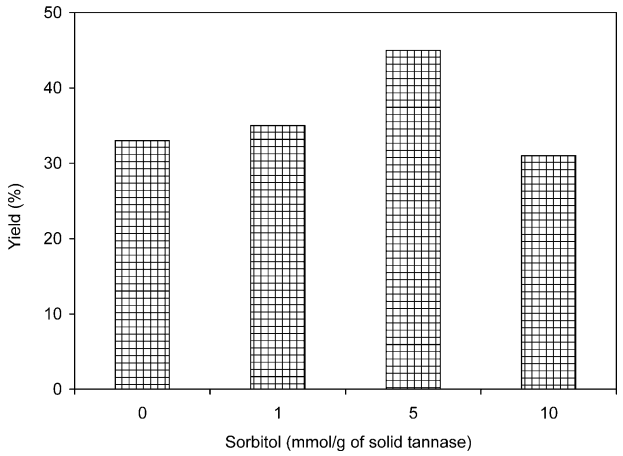

Figure 3. Effect of addition of sorbitol. The tannase powder (5.0 mg) was lyophilized in 100 mM citrate buffer, pH 6.0 and the varied amounts of sorbitol as shown in the figure. The solid powders thus obtained were used for transesterification.

on addition of the optimum amount of 1% water (data not shown).

In conclusion, tannase from *Aspergillus niger* van Teighem can be successfully used with *n*-propanol itself as the organic media to achieve a reasonable product yield of about 86% for propyl gallate under the optimized conditions. While lipases and proteases have been extensively used for synthesis in nonaqueous media, other enzymes been explored much less for this purpose. This work shows that given state-of-art in use of enzymes in nonaqueous media,[4c] it should be worthwhile to explore a wider range of enzymes for synthesis in nonaqueous media.

Acknowledgements

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6. HPLC analysis was performed on Beckman gold system equipped with reverse phase C_{18} column. Ester and acid analysis was carried out after 48 h. Solvent system/mobile phase comprised of water: methanol: acetic acid in the ratio of 65: 35:0.01 at the flow rate of 1 mL min^{-1}(isocratic) for 30 min. Injection volume: 50 μL. Detection: 274 nm. The reaction product was calculated from the standard for propyl gallate (Sigma Chemical Company, USA) plotted as concentration (mM) versus peak area.
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11. Immobilization of tannase on Celite-545: 5 mg of the tuned tannase powder was dissolved in 5 mL of 100 mM citrate buffer, pH 6.0. This solution was lyophilized. The total solid obtained was dissolved in 300 μL of distilled water. This enzyme solution was added to 100 mg Celite. The thick paste was vortexed, and lyophilzed. The entire solid obtained with 100 mg Celite was used in the reaction.