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One-pot bio-synthesis of propyl gallate by a novel whole-cell biocatalyst

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ABSTRACT

Propyl gallate has an excellent antioxidative capacity and some pharmaceutical potentials. In order to examine the feasibility for one-pot bio-synthesis of propyl gallate catalyzed by a whole-cell biocatalyst in organic media, a whole-cell biocatalyst of *Aspergillus niger* was prepared and utilized to catalyze the transesterification with tannic acid as a raw material. Furthermore, both the catalytic system and the reaction mode were optimized to further improve the conversion rate of substrate. The result shows that a promising conversion rate, 43%, was achieved by the pH-tuned mycelium-bound tannase. The rate is over than or very close to that achieved by isolated tannase. The study on reaction mode indicates that the simulated continuous catalysis is the most suitable to the transesterification as compared to batch catalysis and batch catalysis coupled with product separation. Accordingly, the one-pot bio-synthesis of propyl gallate by the novel whole-cell biocatalyst has such three advantages as easy operability of the biocatalyst, high efficiency of reaction mode, and the abundance of the natural raw material, which will contribute to constructing an efficient and eco-friendly method for one-pot synthesis of propyl gallate in an economical and ecological manner.

1. Introduction

Propyl gallate (PG), a gallic acid ester, has an excellent antioxidative capacity, and thus it is usually used as food antioxidant [1]. As a synthetic antioxidant, it can effectively improve the stability of biodiesel toward oxidation [2] and raise the oxidation onset temperature (OOT) [3]. Otherwise, PG also has such potential pharmaceutical properties as an antitumoral effect and an antinociceptive activity [4–8].

Presently, PG can be commercially synthesized by a traditional chemical catalysis means with such defects as high energy consumption and high corrosive environment (caused by concentrated sulfuric acid). Owing to the reaction conditions being mild, biocatalytic method has been applied in PG production instead of the chemical means. There are two routes (i.e. two-step biocatalysis and one-pot biocatalysis) for transforming tannic acid (TA) into PG in biocatalytic method. As compared to the two-step catalysis (the first step: TA is hydrolyzed into gallic acid. The second step: the gallic acid is esterified into PG), one-pot synthesis of PG by transesterification catalyzed by a microbial tannase will be more suitable to commercial manufacture in view of few steps, low cost, and high efficiency. The relevant studies have been reported. For example, Gaathon et al. [9] and Fernandez-Lorente et al. [10] used an isolated tannase as a biocatalyst to catalyze the transesterification reaction for PG production in a reverse micelle system and in a diphase system consisted of water and 1-propanol, respectively. Also, a novel bio-imprinted tannase which can catalyze the one-pot synthesis of PG well was developed in our previous researches [11,12]. However, the cost of the tannase in its preparation process including purification from culture broth and immobilization on a carrier has been one of the main obstacles to industrial application. In comparison with isolated enzymes, whole-cell biocatalysts (WCB) can be more readily and inexpensively prepared [13]. Besides, WCB unlike isolated enzyme can free disperse in organic solvent, and thus can be directly employed in organic phase. All these advantages meet with the requirements for industrial manufacture. Therefore, the application of WCB has been brought into focus [13–18]. But, it has not been seen that WCB is utilized to catalyze the transesterification from TA to PG till now.

In this study, WCB of *Aspergillus niger* was prepared and utilized to catalyze the direct synthesis of PG from TA in anhydrous media. This work aims to examine the feasibility to straightforwardly use the WCB as a biocatalyst for one-pot synthesis of PG

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in organic media. Subsequently, the catalysis system (i.e., solvent type, mycelium dosage, water content, and n-propanol content) was optimized to further improve the conversion rate of substrate (CR). Furthermore, the operability to perform the transesterification with the WCB as a biocatalyst in a large scale was explored by comparative studying three different reaction modes of batch catalysis (BC), batch catalysis coupled with product separation (BCCPS), and simulated continuous catalysis (SCC). It is attempted that an efficient and environmental-friendly way will be constructed to produce PG commercially.

2. Experimental

2.1. Materials

A. niger was from industrial microbiology subdivision of Key Laboratory of Ion Beam Engineering, Chinese Academy of Science (CAS), and this strain used in the present work is a desired mutant with high yield tannase, which was screened from millions of strains implanted by N^+ ion beam. PG (HPLC grade) was purchased from Sigma Co., USA. The other reagents were obtained from Sinopharm Chemical Reagent Co., Ltd. (SCRC), China, and they are of an analytical grade.

2.2. Cultures

The strain was preserved on the PDA medium (composition (g/L): 2.0% glucose, 20% potato extract, and 2.0% agar) at 4 °C. The fermentation medium consisted of rice flour, 5% (g/L); $(NH_4)_2SO_4$, 1%; $MgSO_4$, 0.03%; KH_2PO_4, 0.3%; $CaCO_3$, 0.5%; TA, 2%.

2.3. Preparation of mycelium-bound tannase

A volume of 2 mL spore solution (5×10^7 spores) was inoculated in 50 mL fermentation medium in 250 mL conical flask, and then incubated for 80 h at 30 °C and 180 rpm. The ferment broth was isolated by a three layer of gauzes, and the filter residue was washed by distilled water three times. Subsequently, the mycelium was frozen overnight and lyophilized by a freeze dryer (VirTis, SP Scientific USA) for 24 h. This dry mycelium was defined as WCB.

2.4. Modification of mycelium-bound tannase

Three modifications were designed as following:pH tunning: 0.1 g dry mycelium was submerged in 5 mL 90 mM pH 5.0 citrate buffer, and the mixture was shook up for 5 min.pH tunning-interfacial activation: 0.1 g dry mycelium plus 0.15 mL 10% Triton X-100 were mixed with 5 mL 90 mM pH 5.0 citrate buffer, and the mixture was shook up for 5 min.pH tunning-cryogenic protection: 0.1 g dry mycelium, 0.625 mL 1000 mM mannose and 12.5 μL 200 mM magnesium ions were mixed with 5 mL 90 mM pH 5.0 citrate buffer, and the mixture was shook up for 5 min.

CK: 0.1 g dry mycelium was submerged in 5 mL distilled water, and the mixture was shook up for 5 min.

All the mixtures aforementioned were maintained without agitation for 30 min at ambient temperature, and then they were frozen overnight and lyophilized for 24 h. The lyophilized powders were stored at 4 °C until use.

2.5. Enzymatic reaction

A dose of 0.1 g WCB was added in the catalytic system composed of 50 mg TA, 0.15 mL pH 5.0 90 mM citric acid buffer, 1.5 mL n-propanol, and 18.35 mL hexane in 50 mL conical flask with a sealed plug. The transesterification reaction was performed for 24 h at 40 °C and 200 rpm. In this reaction, CR denotes the mole percent of TA completely transformed to PG ($C_0 - C_t$) relative to the total dose of TA (a fixed concentration, C_0) before the equilibrium of the reaction. The reaction rate can be estimated as the following:

$$v = \frac{C_0 - C_t}{t} = \frac{(C_0 - C_t)C_0}{tC_0} = \frac{CR \times C_0}{t}$$

$$\text{If} \quad k = \frac{C_0}{t}, \quad v = k\text{CR}$$

where v is the reaction rate of transesterification catalyzed by WCB, which can refer to the transesterification-catalyzing capability of the enzyme. t is a fixed reaction time before the equilibrium of the reaction. C_0 and C_t are the initial concentration of TA and the residual concentration of TA in a fixed reaction time, t, respectively.

In consideration of C_0 and t being constants v is proportional to CR. Therefore, CR can be used to estimate the catalytic capability of WCB. All experiments were performed in duplicate unless stated otherwise.

2.6. Effect of organic solvent on the transesterification reaction

A volume of 18.35 mL organic solvents with varying polarity (i.e. n-propanol, hexane, petrolether, and benzene) as reaction media were added in 50 mL conical flasks containing such other components as 0.1 g WCB, 50 mg TA, 0.15 mL pH 5.0 90 mM citric acid buffer, and 1.5 mL n-propanol (distilled water was chosen as a control solvent), respectively. The transesterification reaction was performed at 200 rpm and 40 °C for 24 h. The solvent effect on the reaction was evaluated by analyzing the difference in CR between organic solvents and distilled water. All organic solvents had been dried over 3 Å molecular sieves for 72 h.

2.7. Reaction modes

BC: A dose of 0.15 g WCB was added in catalytic system composed of 50 mg TA, 0.3 mL pH 5.0 90 mM citric acid buffer, 2.5 mL n-propanol, and 17.2 mL hexane.

BCCPS: A dose of 0.15 g WCB was added in catalytic system (same as that in BC). All of the old reaction system containing bulk product was replaced by the equivalent fresh reaction system without TA every 24 h.

SCC: A dose of 0.15 g WCB was added in catalytic system (same as that in BC). All of the old reaction system containing bulk product was replaced by the equivalent fresh reaction system with 20 mg TA supplement every 24 h.

All of the transesterification reactions were carried out for 120 h at 40 °C and 200 rpm.

2.8. Preparation of calibration curve for PG and assay of propyl gallate

A series of PG (HPLC grade) solutions with various concentrations were prepared with methanol (HPLC grade) as s solvent, and their corresponding integral areas were determined by HPLC (Waters 600, Waters, USA) with Phenomenex C18 column with 250 × 4.60 mm, 4 μm at 35 °C. The calibration curve for PG ($y = 8.48 \times 10^{-8}x$, $R^2 = 0.9999$) was plotted with the concentrations of PG and their corresponding integral areas as the vertical axis and the horizontal, respectively (shown in Fig. 1). The integral area of sample was determined by HPLC under the same conditions mentioned above, and then its concentration was calculated by the calibration curve. All of samples including standard substances and samples with unknown concentration were filtrated by organic membrane (Φ 0.9 μm). The mobile phase consists of 50% methanol, 50% pure water, and 0.01% acetate acid. A dose of 0.02 mL sample was detected at 274 nm at a flow rate of 1 mL/min.

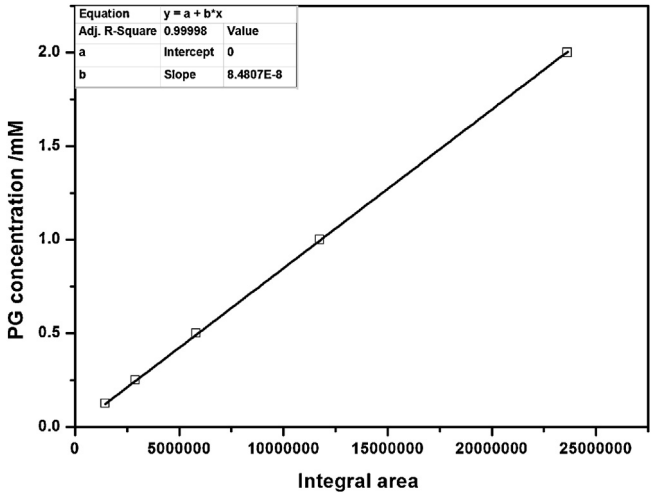

Fig. 1. Calibration curve of propyl gallate for HPLC.

3. Results and discussion

3.1. Effect of mycelium-bound tannase on the transesterification

A. niger was improved in tannase yield by successive N^+ ion beam implantation in our previous work [19]. An excellent mutant J-T18, which is 5-time that of the original strain in tannase yield, was achieved, and its WCB has a promising specific activity of 44 IU/g (assayed by the method reported by Sharma [20]). The WCB was treated by three methods including pH tunning, pH tunning-interfacial activation, and pH tunning-cryogenic protection, respectively, to further enhance its transesterification-catalyzing activity. The result shows that all of the three treatments can dramatically improve the activity of WCB, in which the WCB modified by pH tunning and pH tunning-cryogenic protection has appropriate 2.3-time CR over that of CK (shown in Table 1). The pH-tuned WCB can more effectively disperse in organic media than the one treated by pH tunning-cryogenic protection because the hydrophilic protector, mannose as an interlink, leads to the WCB converging in organic media. Consequently, the pH-tuned WCB was determinded as a biocatalyst for the transesterification due to its perfect dispersibility in favor of transference of substrate and product. In addition, the dose effect of the WCB on the reaction efficiency of the transesterification was investigated by adding varying dose of WCB (0.05–0.30 g) in the same reaction system. The result shows that the CR increases with growth of the dosage. Notedly, the CR rises fast in the dosage less than 0.15 g (show in Fig. 2a). On the other hand, it is found from Fig. 2b that the catalytic efficiency of the WCB at 0.15 g is the optimum. Accordingly, the dosage of the WCB was determined as 0.15 g in the further investigation. Hyper-activation of WCB by pH-tunning is ascribed to the "memory" characteristic of enzyme [21–23]. Based on this, the conformation of enzyme molecules can be regulated by pH tunning in aqueous phase and be fixed in organic phase. In this work, a favorable conformation with high activity was induced by pH tunning with pH 5.0 citric acid buffer because the optimal pH value of tannase ranges from 4 to 6. In comparison with WCB, isolated tannase is more sensitive to ambient temperatures [12]. The fine difference between the CRs of WCBs achieved by pH tunning and pH tunning-cryogenic protection implies that intracellular enzymes are protected from the external environment so that they are more stable in the long-term than isolated enzymes. In this present work, WCB unlike isolated tannase was manufactured well only by a simple separation process and a pH tunning procedure. This suggests that the preparative protocol required for WCB is simpler than that of isolated tannase. The simplicity gives rise to the WCB being commonly applied in commercial synthesis. Furthermore, rapid advances in biotechnology have greatly enhanced the availability of WCB. E.g., the recombinant DNA technique has enabled to overproduce all sorts of desired enzymes in various heterologous hosts (microorganism, plant, and animal cells), and this technique has even made it possible to modify metabolic pathways in the host cells so as to develop some

Table 1
Effects of different treatments on the catalytic capability of mycelium-bound tannase and its discrete state in reaction system.

Treatments	CR/% (SD)	Discrete state of mycelium
pH tunning	11.96 ± 0.30	Diffusion
pH tunning-interfacial activation	10.64 ± 1.41	Diffusion
pH tunning-cryogenic protection	12.00 ± 0.31	Aggravation
CK	5.2 ± 0.41	Aggravation

The reaction conditions were seen in the part of "Enzymatic reaction" given in Section 2.

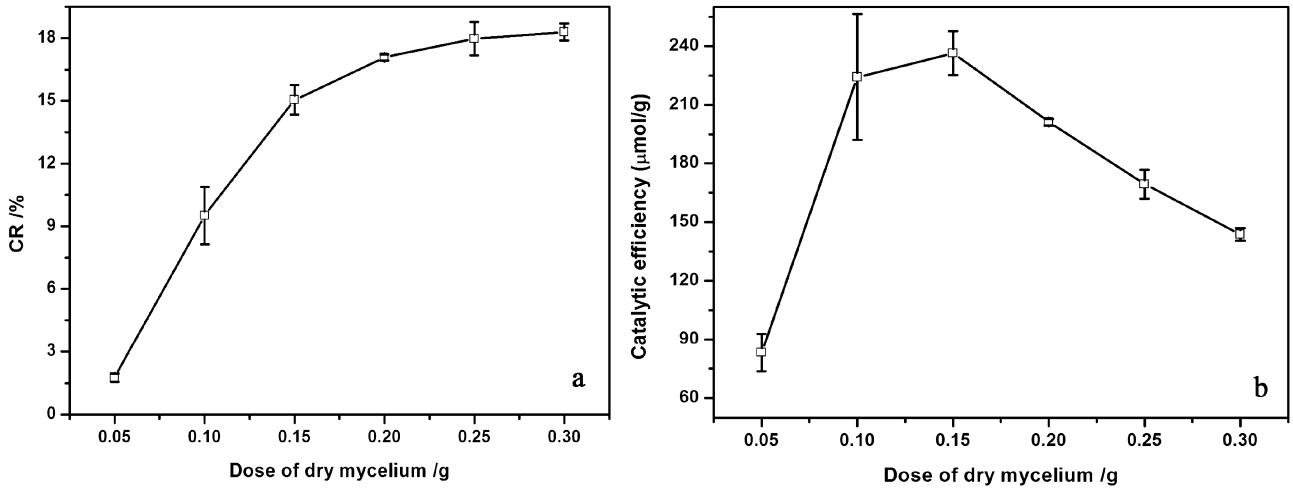

Fig. 2. Effect of mycelium dose on conversional rate of substrate (a) and its catalytic efficiency (b). (a) Different dose of WCB (from 0.05 to 0.30 g) was applied to catalyze the transesterification. (b) The effects of WCB dosage on reaction efficiency have been estimated by comparing their catalytic efficiencies, which was calculated by the ratio of the achieved CR to the corresponding WCB dosage. Reaction conditions: 50 mg TA, 0.15 mL pH 5.0 90 mM citric acid buffer, 1.5 mL n-propanol, and 18.35 mL hexane, incubated at 40 °C and 200 rpm for 24 h.

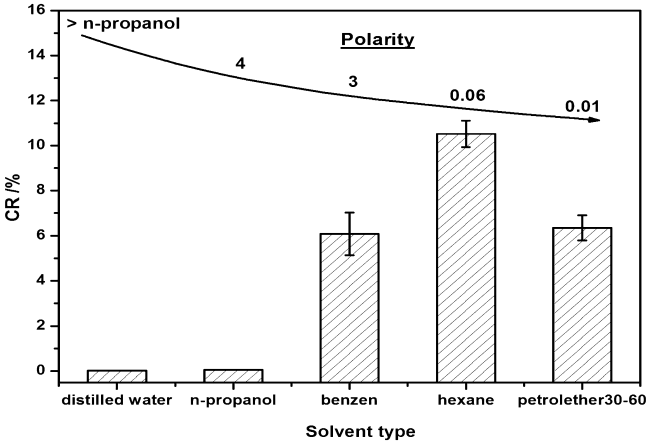

Fig. 3. Effect of solvent type on the transesterification reaction. In this diagram, the variation trend of the line with arrow exhibits the polarity change of the organic solvents. The data above the line refer to the polarity values of the corresponding organic solvents on the horizontal axis (the data referred from the polarity chart of organic solvent). Reaction conditions were seen in the part of "Effect of organic solvent on the transesterification reaction" given in Section 2.

new enzymatic synthesizing processes for specific use of WCB. Even so, such problems as transference of substrates across the cell membrane, accumulation of product, or existance of side reactions, which has hindered the industrialization of whole-cell biocatalysis [24]. These problems need to be solved in future work.

3.2. Effect of solvent on the transesterification reaction

In this present work, the effect of solvent on the transesterification reaction was studied by using four solvents with different polarity (i.e. n-propanol, hexane, petrolether, and benzene) as a reaction media, respectively. The result indicates that the CR rises as the order: hexane > petroleum ether (30–60 °C) > benzene > n-propanol > distilled water (control solvent). This suggests that the enzymatic activity increases with the polarity of reaction media decreasing, but the activity will decrease when the polarity of solvent is reduced to a certain value (shown in Fig. 3). Accordingly, hexane was applied as reaction media for further study.

To our best knowledge, non-polar solvents are more favorable to transesterification or esterification as compared to hydrolysis. In detail, the organic solvents with a log P between 2 and 4 are better for enhancing catalytical performance of enzyme, but organic solvents with a log $P < 2$ cannot [25]. It is likely that non-polar solvents, such as hexane ($\log P = 3.5$), cannot strip off the essential water around enzyme molecules, and thus the active conformation of the enzyme is retained [26]. Also, it has been reported that the effect of organic solvents on enzymatic activity is depended on not only the log P, but also the functional groups and molecular configuration of organic solvents [27]. Besides, the polarities of substrate and product also have a dramatical effect on the catalytic efficiency of enzyme [25]. In this research, hexane as a hydrophobic solvent cannot only maintain higher effective concentration of hydrophilic substrate (TA) in microaqueous layer around protein molecules, but also removes the feedback inhibition of hydrophobic product (PG). This promotes the reaction equilibrium to shift from hydrolysis to synthesis and also is helpful for improvement of the CR and separation of the product.

3.3. Effects of water content and n-propanol content on the catalytic capability of mycelium-bound tannase

Essential water is greatly crucial for emergence of enzymatic activity in organic media. The effect of water content on the CR was investigated by adding 0.15–0.5 mL pH 5.0 90 mM citric acid buffer in the reaction system, and the result is shown in Fig. 4a. The CR rises with the water content increasing up to 1.75%, while the content continues to increase it glides slowly. This suggests that water content in organic biocatalytic system dramatically affects the catalytic activity of the enzyme. It can be due to that an enzyme with a certain flexible conformation only can exhibit a good catalytic performance. Generally, the conformation of an enzyme in organic media is rigid. The pH-tuned WCB has a desired conformation but not enough flexibility in pure organic phase. Therefore, addition of appropriate water in this reaction system can form the hydration shell around enzyme molecule which maintains the enzymatic structure to be more stable and flexible [28]. It is noted that a lower water content leads to the formation of the shell incompletely, but a higher water content could result in the conformation of enzymatic active center changing so easily that its specific activity decreases. Otherwise, the requirement of water activity is usually different for various reactions catalyzed by the same enzyme. With regard to tannase, high water activity will be in favor of hydrolysis, whereas lower water activity will be better for esterification or transesterification [29–31]. It was found from our previous study that the optimum of water content is 1.5% in the transesterification catalyzed by isolated tannase [32]. On the other hand, a polar substrate has a significant effect on the biocatalytic performance of an enzyme. The dependence of the CR on n-propanol content from 5 to 22.5% (v/v) was investigated. Fig. 4b indicates that the CR grows as the content of n-propanol rising up to 12.5%, at which the maximal CR of 21% was achieved. Meanwhile, it was found from the clarity of reaction system that the reaction solution was great clear at 5–12.5% n-propanol concentration. On the contrary, the reaction solution became turbid as the concentration adding up to more than 12.5%. This may be the reason why a low concentration of n-propanol can completely dissolve in the reaction system, but a higher concentration of n-propanol cannot. The increment of n-propanol dosage can increase the effective concentration of the substrate at a lower concentration from 5 to 12.5% (n-propanol as a substrate), while at a higher concentration above 12.5%, the excessive n-propanol (as a solvent) can despoil the essential water around enzyme molecules so as to disrupt hydration membrane of the enzyme [33]. Therefore, it is greatly important to make n-propanol in the reaction system located in an optimal equilibrium between as a substrate and as a solvent. The same observation has been reported by Yu [33], but Sharma et al. have given completely opposite findings [34]. With a view to the effects of water and polar substrate on enzyme and the intersolubility of substrate-product-reaction media, there will be a good opportunity that the specific activity of the WCB can be improved by optimizing the reaction system based on studying the interrelationship of substrate-product-bulk solvent in reaction system.

3.4. Effect of production mode on the catalytic capability of mycelium-bound tannase

The catalytic effiency of WCB in the transesterification was further improved by comparative studying such three reaction modes as BC, BCCPS and SCC. Fig. 5a shows that the maximum CR of 43% was obtained by BCCPS, followed by a CR of 33% achieved by SCC. The CR in BCCPS is close to that reported by Fernandez-Lorente [10], where a CR of 45% was obtained using immobilized tannase in the water–propanol (1:1) reaction system. In addition, Fig. 5b indicates that SCC is the best in PG yield among the three reaction modes, and the yield in SCC is 2-fold that in BC and 1.33-fold that in BCCPS. This suggests that optimization of reaction mode can promote not only the CR but also the production efficiency. As a matter of fact, SCC is similar to continual catalysis reaction (CC), which has many evident advantages, such as its continuity of operation, and

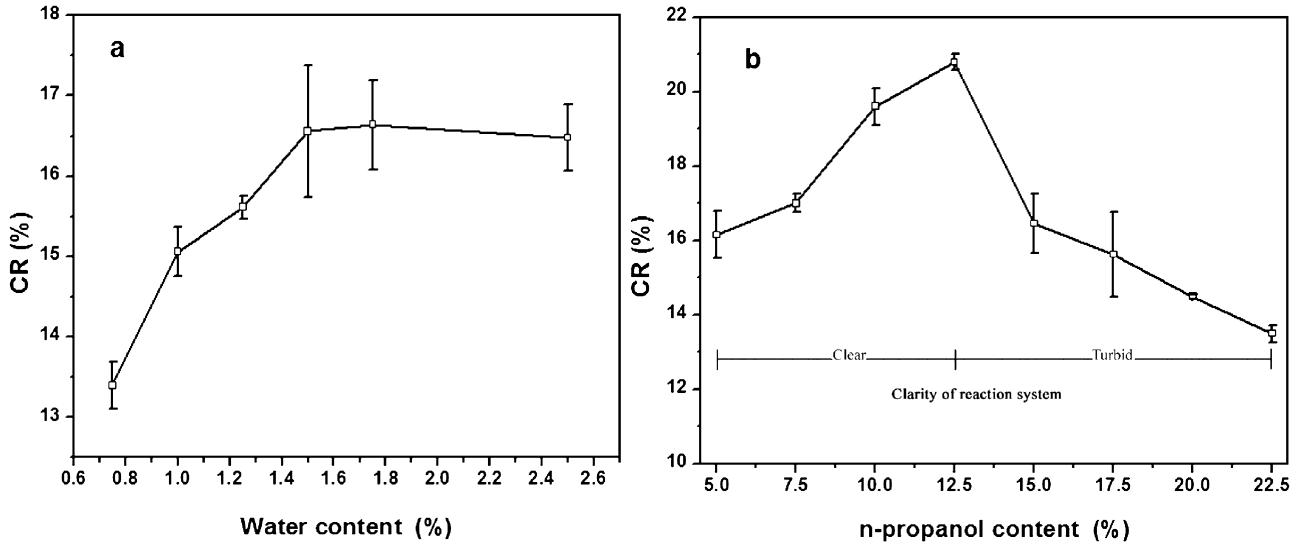

Fig. 4. Effects of water content (a) and n-propanol content (b) on the catalytic capability of mycelium-bound tannase. (a) Reaction conditions: 0.15 g WCB, 50 mg TA, 0.15–0.5 mL pH 5.0 90 mM citric acid buffer, 1.5 mL n-propanol, and 18–18.35 mL hexane, incubated at 40 °C and 200 rpm for 24 h. (b) Reaction conditions: 0.15 g WCB, 50 mg TA, 0.3 mL pH 5.0 90 mM citric acid buffer, 1–4.5 mL n-propanol, and 15.2–18.7 mL hexane, incubated at 40 °C and 200 rpm for 24 h. In this diagram, the words "Clear" and "Turbid" present the clarities of reaction systems with 5.0–12.5% and 12.5–22.5% n-propanol content, respectively.

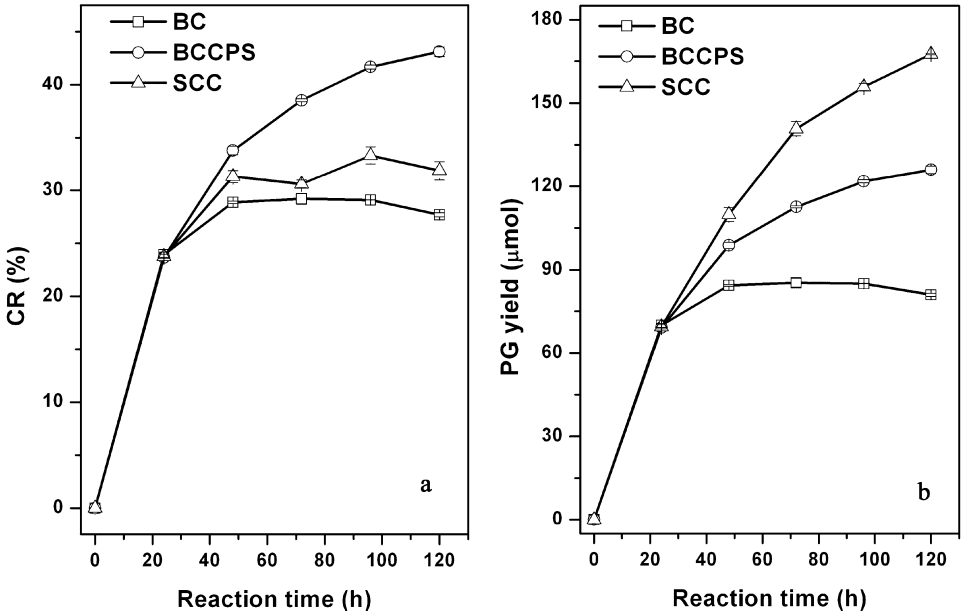

Fig. 5. Effect of reaction mode on conversional rate of substrate (a) and propyl gallate yield (b). The reaction system and reaction conditions were seen in the part of "Reaction modes" given in Section 2.

an economic optimization in terms of input flow rate of substrate, output flow rate of product, as well as product separation in industrial application. In consideration of the advantages of WCB, it is speculated that the WCB will be hopefully exploited in commercial production of PG. In view of the great difference between TA and PG in the molecular weight, to develop a circulation membrane reactor in favor of SCC will be greatly vital for efficient and continuous transformation of TA into PG coupled with product separation in a large scale.

4. Conclusions

In summary, it is noted that the promising CR of 43% achieved by the pH-tuned WCB is over than or very close to that obtained by isolated tannase. This suggests that straightforward use the WCB as a biocatalyst in transesterification-synthesis of PG will be feasible. As for reaction mode, SCC is the most suitable to the transesterification. Besides, TA present in a variety of agro-forestry residues as a raw material is remarkably abundant [35]. Based on this, it is expected that the one-pot bio-synthesis of PG catalyzed by the WCB in organic media will exhibit enormous economical and ecological values.

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