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Three-Phase Partitioning for Purification of Laccase Produced by *Coriolopsis trogii* under Solid Fermentation

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ABSTRACT

In order to explore separation and purification methods of *Coriolopsis trogii* laccase production by solid fermentation, we used a three-phase separation technique and wherein the main factors (ammonium sulfate concentration, tertbutanol concentration, temperature and pH) were optimized. The results showed that when the three-phase partitioning optimum conditions were as follows: Ammonium sulfate concentration was 40%, tertbutanol water phase volume ratio of 1:1, the temperature is 20°C, pH 5, laccase purification factor and recovery can be up to 20 times and 75%, respectively, SDS-PAGE electrophoresis showed that the purified protein is substantially a single band and a molecular weight of about 38 kD, smaller than the known fungal laccase molecular weight; the results prove that the three-phase separation from the crude enzyme solution *Coriolopsis trogii* solid obtained after fermentation relatively pure laccase, eliminating the need for complex chromatography step, the method is simple and low cost, for a lot of separation and purification of laccase provide practical foundation.

Key words: *Coriolopsis trogii*, laccase, three-phase partitioning, purification

INTRODUCTION

Laccase (Laccase, EC 1. 10. 3. 2) is a multi-copper ion oxidase, its pure enzyme protein exhibits blue and ascorbic acid oxidase, bilirubin oxidase, tyrosinase and plasma ceruloplasmin similar nature, is known as the blue multi-copper oxidase (blue multicopper oxidase) family cluster, which contains four copper ions and relatively close in structure and function (Farver *et al.*, 2011; Mate *et al.*, 2013). Laccase oxidizes most phenols and some aromatic compounds, meanwhile, with the four electron transfer, molecular oxygen reduction into water. Laccase first in a sumac sap discovered by Japanese scholars and identified as a metal oxidase (Farver *et al.*, 2011). There are three main types of laccase: Plant laccase, fungal Laccase and bacterial laccase but *Coriolopsis trogii* laccase most studied. Laccase mostly monomeric form, in the form of aggregate rarely exist, its molecular weight is generally between 52-110 kDa, currently, there is not found below the 40 kDa but there is more than 110 kDa laccase protein was found (Mate *et al.*, 2013; Silva *et al.*, 2012). Now has been reported that, about six categories of substrates can be laccase catalyzed oxidation, including a variety of phenols and other non-phenolic substrates and so on. Laccase in the industrial field biosensors, chemical synthesis, food, environment, etc. have a certain application processing (Fernandez-Fernandez *et al.*, 2013; Ba *et al.*, 2014). Now found that laccase catalytic reaction formation of free radicals has a killing effect on cancer cells, can be prepared anticancer drugs (Wu *et al.*, 2014). In addition, it does also inhibit reverse transcriptase for AIDS virus (HIV virus) and in immunoassays alternative peroxidase as a new marker enzyme (Sabiiti *et al.*, 2014), thus, in the medical application laccase also shows good prospects.

With more and more applications of laccase, its demand is also growing and large-scale extraction and purification become a big problem in the industrial production of this enzyme. The main application of protein separation and purification by column chromatography, usually require multiple column combinations, step chromatography and optimized conditions, the entire procedure is more complicated, while a variety of chromatography media is expensive, which results in the high cost of producing laccase, so that the further application is affected, so to find a simple, efficient and convenient method of extracting the enzyme is very necessary. Three-phase partitioning referred TPP is a simple and effective separation technique, by an organic solvent and salt (e.g., tert-butanol and ammonium sulfate) added to the crude enzyme solution, after processing a certain time, the crude enzyme solution will form a three-phase mixture, the upper layer is an organic phase (containing most of the non-polar compound impurities), the lower layer is mainly water phase (containing polar compound impurities), the intermediate layer is a protein class precipitation (Gagaoua *et al.*, 2014; Avhad *et al.*, 2014). The TPP protein purification process can be applied simultaneously upstream and downstream, can also be used as a single-step purification method, has been reported as an important alternative to enzyme concentration and purification (Wang *et al.*, 2012b). Currently, TPP paint applied to the extraction of the enzyme protein also rarely reported and the application of TPP protein extraction and purification of laccase from white rot fungi solid fermentation broth has not been reported. In this paper, by changing the phase separator in the ammonium sulfate concentration, pH, temperature and t-butanol concentration factor to optimize laccase phase separation conditions, to obtain a higher purity of laccase, for large-scale fermentation product from solid extraction of the enzyme provides a guideline and reference.

MATERIALS AND METHODS

Strain (*Coriopsis trogii*) screening and identification by the laboratory

Reagents and instruments: The 2, 2'-even nitrogen-double -3-ethyl-6-sulfonic acid benzothiazol-pyrroline) (ABTS), the Sigma company, USA; bovine serum albumin, Beijing Ding domestic products; low molecular weight proteins belong to Beijing Kang Marker century of biotechnology products, other reagents are analytical grade. UV-visible spectrophotometer (WFZ UV-2000), desktop high-speed refrigerated centrifuge (H2050R), large-capacity high-speed refrigerated centrifuge (GL-10 MD) and so on.

Enzyme production medium: The 20 g bran, 60 mL distilled water and mix into the 500 mL triangle.

Laccase activity assay (Qi *et al.*, 2014)

Determination of protein concentration: Determination of protein concentration is based on reference to bovine serum albumin as the standard protein.

Preparation of crude enzyme solution: Collecting the culture to the solid culture after 20 days, per 500 mL shake flask was added 200 mL distilled water, soak for 24 h and then filtered with a double filter paper, the filtrate was collected, centrifuged at 4°C 10000 g 30 min, the supernatant was collected. 80% saturation of ammonium sulfate precipitation supernatant, to stand overnight at 4°C 10000 r min⁻¹ centrifugal 20 min, the supernatant was discarded, the precipitate was dissolved in distilled water, dialyzed overnight and the resulting dialysate is the crude enzyme solution.

Optimization of the three-phase separation conditions

Ammonium sulfate concentration on the three-phase separation of comparison: At room temperature, added ammonium sulfate in 3 mL of the crude enzyme solution, the final concentration of ammonium sulfate were 20, 30, 40 and 50%, added an equal volume of t-butanol mixed, placed in 25 incubator for 1 h, 12000 r min⁻¹ centrifugation 10 min, collect the intermediate phase, with 9 mL of distilled water to dissolve after activity and protein concentration was measured according to standard methods.

t-butanol concentration in comparison with the three-phase separation: Other conditions are the same as above, added 30% ammonium sulfate concentration in 3 mL of the crude enzyme solution and t-butanol was added in various proportions to the crude enzyme solution, in the same manner as described above were collected mesophase determination of enzyme activity and protein concentration.

Temperature in comparison with three-phase separation: Other extraction conditions and the above agreement and then change the extraction temperature, in the same manner as described above were collected mesophase determination of enzyme activity and protein concentration.

pH in comparison with three-phase separation: Change the pH value, other conditions remain unchanged, in the same manner as described above were collected mesophase determination of enzyme activity and protein concentration.

Electrophoresis: The SDS-PAGE sees Ref. (Liu *et al.*, 2008), separating gel concentration was 10%, shaker stained SDS-PAGE with Coomassie Brilliant Blue R 250 2 h; PAGE activity staining according to (Qian-Guo and Jiang, 2012) slightly modified, using a substrate for guaiacol.

RESULTS

Ammonium sulfate concentration in comparison with the three-phase separation: As shown in Fig. 1, with the improvement of ammonium sulfate concentration, purification factor and activity recovery will be improved, from 30-40% purification rate and recovery rate increase is the largest. Wherein when the ammonium sulfate concentration is 40%, Purification factor and recovery are 3.3 times and 25%, when ammonium sulfate concentration is 50%, purification and recovery multiples are 3.4 times and 35%, although recovery has increased but the purification

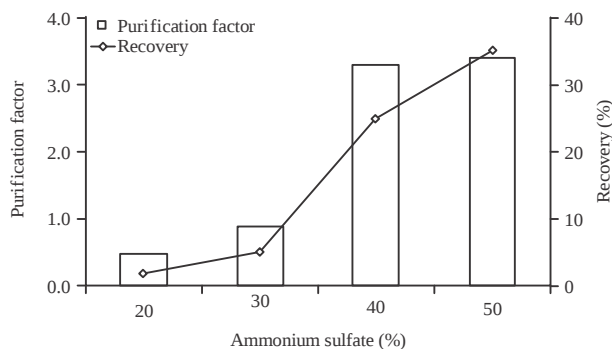


Fig. 1: Ammonium sulfate concentration in comparison with the three-phase separation

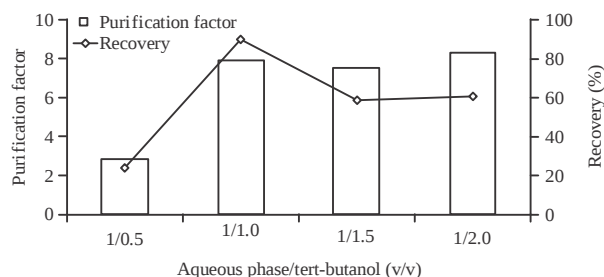


Fig. 2: Tert-butyl alcohol concentration in comparison with three-phase separation

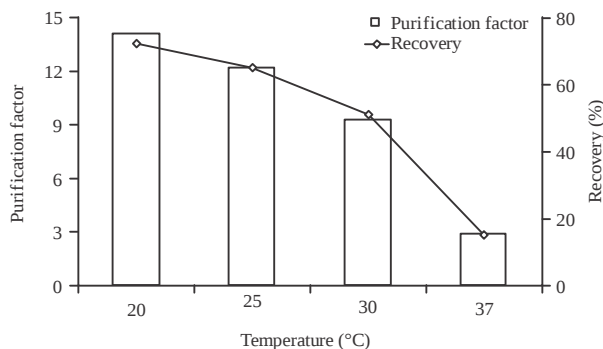


Fig. 3: Temperature in comparison with three-phase separation

factor is essentially the same, means to improve protein recovery, this is due to other contaminating proteins precipitated caused, from the purity of the target protein and the economic cost considerations, should be selected as the best 40% of the ammonium sulfate concentration.

Tert-butyl alcohol concentration in comparison with three-phase separation: As shown in Fig. 2, with the ratio of t-butanol and the aqueous phase is gradually increased, purification factor gradually increase but recycling lead increased and then decreased. Wherein when t-butanol and aqueous phase was 1/1.0, the recovery and purification factor were the highest, respectively, 90% and 8 times, therefore, choose 1/1.0 of the water and tert-butyl alcohol volume as the optimal separation conditions.

Temperature in comparison with three-phase separation: As shown in Fig. 3, with the improvement of the temperature gradually, purification rate and recovery rate gradually reduced. Wherein when the temperature is 20°C, the recovery and purification factor are the highest, reaching 72% and 14 times, so choose the optimum temperature is 20°C for extraction conditions.

pH in comparison with three-phase separation: As shown in Fig. 4, with the increase of pH gradually, purification factor and recovery of lead increased and then decreased gradually. Wherein, when the pH is 5, the recovery and purification factor are the highest, reaching 86% and 15 times, so choose the optimum extraction conditions pH 5.

Comparison of all single optimal conditions and optimal conditions: As shown in Fig. 5, after optimization of crude enzyme liquid by the concentration of ammonium sulfate, tert-butyl alcohol concentration, temperature and pH, purification factor and recovery overall

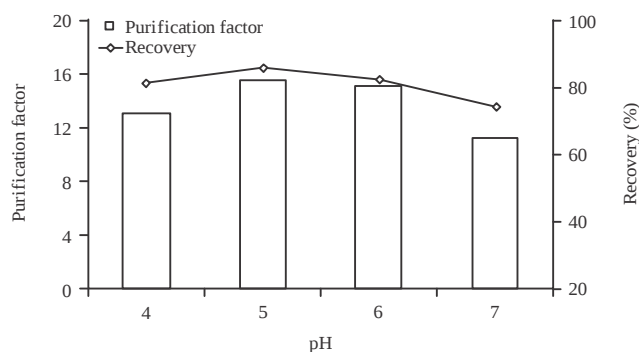


Fig. 4: pH in comparison with three-phase separation

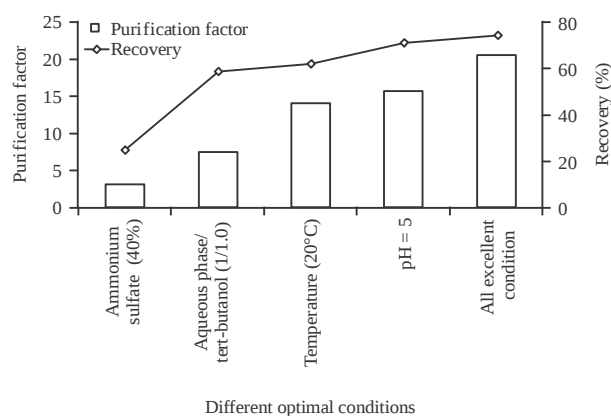


Fig. 5: Comparison of all single optimal conditions and optimal conditions

upward trend, after the comprehensive optimization, purification factor and recovery is greatly improved, with respect to ammonium sulfate concentrations in terms of optimization, after optimization of recovery increased nearly 25-fold, purification factor increased by nearly seven times. In a single impact conditions, ammonium sulfate concentration and tert-butyl alcohol concentration on the influence of the phase separation effect is small, the greater effect of temperature and pH on the three-phase separation effect.

Activity electrophoresis of laccase and SDS-PAGE: As shown in Fig. 6 (left), the extracted under different conditions to optimize the activity of laccases electrophoresis, the original thick enzyme fluid has three isozymes (left lane 1). Among them, the third stripe color darker, indicating that the content may be higher; original crude enzyme solution after three-phase separator, only shows one active band, show two other less paint isoenzyme content in the extraction process with the hybrid protein to remove; by comparing the main laccase (Article) color, original crude enzyme solution deepest, followed by extraction under all excellent condition enzyme solution, although the three-phase separation and extraction process, under all excellent condition, laccase extraction recovery is higher than recoveries single optimized conditions. The optimal conditions in full paint enzymes extracted to SDS-PAGE, as shown in Fig. 6 (right, lane 6), we found that laccase obtained substantially single band, other most of the contaminating proteins are removed and the resulting molecular weight is about 38 kDa.

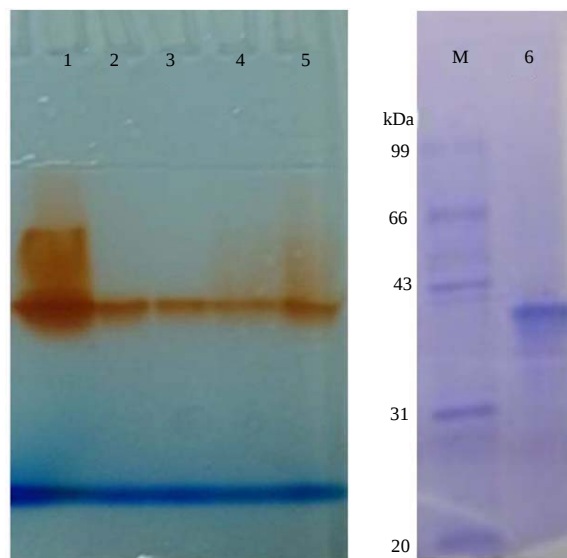


Fig. 6: Activity electrophoresis (Left, different optimal conditions extracted laccase, Lane 1: Crude enzyme, Lane 2: Tert-butyl alcohol, Lane 3: Temperature, Lane 4: pH, Lane 5: Full excellent condition) and SDS-PAGE (right, M: Protein Marker and Lane 6: Extracted laccase in all excellent condition)

DISCUSSION

In this experiment, the crude enzyme solution from *Coriolopsis trogii* solid state fermentation cultures extracted, laccase belong to *Coriolopsis trogii* secretion of extracellular one of lignin degradation enzyme system. The study reported that use of agricultural solid waste as *Coriolopsis trogii* culture medium, while beneficial to induce laccase production, on the other hand is conducive to reducing the cost of enzyme production (Qi *et al.*, 2014). Before carrying out three-phase separation, this paper from *Coriolopsis trogii* solid fermentation culture with distilled water to soak the enzyme solution, then precipitation with ammonium sulfate (80% saturation), by this step can be part of the contaminating proteins and fats and other impurities removed by this step, which is conducive to subsequent further purification. Three-phase separator is a kind of simple separation technique, economic, efficient and simple and easy-to-zoom, can be used as purified proteins or other carbohydrates alternative technology. Many factors influence the effect of three-phase separator, including enzyme protein characteristics, the enzyme protein concentration, pH, ionic strength, temperature, organic solvent type and processing time, it is necessary to study the effects of these factors on the three-phase separation and three-phase separation conditions were optimized (Wang *et al.*, 2007). In the three-phase separation, the salts are usually selected potassium sulfate and sodium chloride but most ammonium sulfate, as the salt on the enzyme activity of the protein is minimized. In addition, because the t-butanol molecules are small, branching structure is rather special but it is not easy to penetrate into the internal structure of the enzyme protein folding, do not cause inactivation of the enzyme protein denaturation, so it was chosen as the organic phase separator solvent (Wang *et al.*, 2007).

The results can be known from Fig. 5, the larger the effect of pH on the three-phase separation effect. Three-phase separation and different traditions of ammonium sulfate precipitation, when in the process of phase separation in solution pH near the target protein isoelectric point, sulfate

anion and cation of the enzyme protein with electrostatic interactions would drop dramatically, leading to a three-phase separation significantly affected (Wang *et al.*, 2007; Bayraktar and Onal, 2013; Wang *et al.*, 2012a). This can be used and the ammonium sulfate concentration to adjust the pH of the crude enzyme solution was precipitated contaminating proteins and then adjusting the pH and the concentration of the ammonium sulfate precipitated protein, so as to achieve the purpose of fractional precipitation. The purified laccase by SDS-PAGE electrophoresis, which shows that the molecular weight of laccase is 38 kDa around and with a single strip, indicating that its purity has been greatly improved. Previous studies have reported that, general molecular laccase between 50-110 kDa (Kallio *et al.*, 2011), rarely found in smaller molecular weight laccase, the present study to give varnish protein molecular weight is about 38 kDa, shows that laccase may be extracted to obtain a novel laccase, about the nature of the laccase, in subsequent experiments, they are subject to further study. After optimization of the three-phase separation conditions, in all excellent condition, we found multiple purification and recovery of up to 20 times, respectively and 75%, this suggests that the three-phase separation method is suitable for the white-rot fungus (*Coriolopsis trogii*) solid state fermentation cultures laccase extract.

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