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Original Research

Phenol Removal from Palm Oil Mill Effluent Using *Galactomyces reessii* Termite-Associated Yeast

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Abstract

Lignin-modifying enzymes have long been used in palm oil mill effluent (POME) treatment to remove the dark brown colour resulting from phenolic contamination. This study investigated a cost-effective industrial application method for optimizing phenol removal from POME using the termite-associated yeast *Galactomyces reessii* obtained from the subterranean termite under laboratory conditions. The yeast was cultured in POME, and the activity of the ligninolytic enzymes (laccase and manganese peroxidase) was monitored by spectrophotometry. Optimal conditions were achieved using a Box-Behnken experimental design. Results demonstrated that *G. reessii* reduced the phenolic compounds in POME by 88.69% with growth in 100% (v/v) POME using 30% (w/v) consortia and 5% (w/v) calcium carbonate ($CaCO_3$) at room temperature 30 ±1°C for seven days. *G. reessii* showed high performance for phenolic removal and decolourization of POME and other industrial wastewaters.

Keywords: optimization, palm oil mii effluent, phenolic compound, termite-associated yeast, Box-Behnken design

Introduction

The global situation in the decrease of fossil fuels and other non-renewable energy sources is now of significant concern. Interest in renewable energy is increasing from this situation. The oil palm (*Elaeis guineen* L.) is known as a sustainable energy source for biodiesel production. It is more versatile than other oil crops and provides a higher yield for less energy input [9]. Highly colored palm oil mill effluent (POME) generates approximately 60% of global crude palm oil [10]. Today the oil palm is an important economic crop in southern Thailand, and in 2016 oil palm plantations covered an area of 720,000 ha. A recent report by the National Science and Technology Development Agency (NSTDA) indicated that Thai agriculturists produced 9 million tons per year of fresh fruit bunches (FFB), with 0.19 tons of POME released from every ton of FFB. Consequently, around 1.026 million tons of dark-colored POME was produced annually. Raw POME is a highly polluting wastewater containing 95-96% colloidal suspensions, 4-5% total solids, and 0.6-0.7% palm oil. The oxidation of phenolic compounds such as lignin and anaerobically degraded products result in the dark

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brown color of POME [7]. Treatment of POME always uses aerobic and anaerobic bacteria. This system can decrease high biological oxygen demand (BOD) from the wastewater, but does not remove the dark brown color. Thus, physical and chemical procedures have been attempted to resolve this problem, but this method has a higher cost per unit volume of wastewater treated than the use of biological systems [10-12].

Lignin-modifying enzymes such as laccase (EC 1.10.3.2) and manganese peroxidase (EC 1.11.1.13) are well-known oxidoreductases used in the decolorization of industrial paper mill and palm oil mill effluents [15]. Laccases are blue multi-copper oxidases that catalyze the monoelectronic oxidation of a broad spectrum of substrates, including diphenols, polyphenols, aminophenols, and amines through a four-electron reduction of oxygen (O_2) to water (H_2O). Multi-copper oxidases are capable of degrading lignin and these are abundant in many white-rot fungi [17]. All wood-decaying fungi – including the white-rot fungi *Trametes versicolor*, *Pleurotus ostreatus*, *Xylaria* sp., and the termite-associated fungus *Termitomyces* sp. – show high potential of lignin-modifying enzyme production [1, 2, 5, 16]. Symbiotic yeasts play an essential role through plant material fermentation in the hindgut of subterranean termites. The two yeast strains, *Debaryomyces hansenii* and *Sporothrix albicans*, were found in the hindgut of the lower termites *Mastotermes darwiniensis*, *Zootermopsis angusticollis*, *Zootermopsis nevadensis*, *Neotermes jouteli*, *Reticulitermes santonensis*, and *Heterotermes indicola* [14].

Our recent study focuses on the subterranean termite-associated yeast *Galactomyces reessii* from para rubber plantations in southern Thailand and reported on its lignin-modifying enzyme activity. The symbiotic yeast was screened for phenol removal activity. We found that the ligninolytic yeast had high ability for phenol removal in POME [3]. In this work, the optimal conditions of phenol removal in POME were investigated using the Box-Behnken experimental design. The ligninolytic yeast was monitored for its phenolic removal and decolourization potential of POME using spectrophotometry.

Experimental

Microorganism and Culture Conditions

The ligninolytic yeast *G. reessii* was obtained for our laboratory on Phatthalung Campus at Thaksin University in southern Thailand. The culture was maintained on malt extract agar (MEA; 20 g/L of malt extract, 20 g/L of dextrose monohydrate, 6 g/L of peptone, and 18 g/L of agar) supplement with 50 g/L of chloramphenicol to avoid bacterial contamination until required for use. For consortium preparation, the yeast on MEA was bored by a cork borer (4.0 mm i.d.). The culture plug was transferred in malt extract broth (MEB) supplement and incubated at 30±1ºC for five days with 150 rpm shaking.

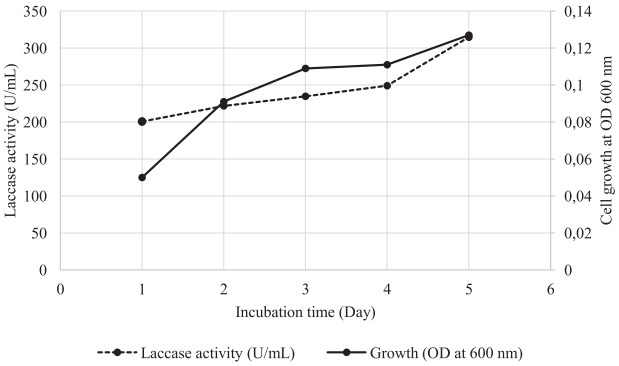

Fig. 1. Growing of *Galactomyces reessii* in raw POME and its laccase activity (U/mL).

POME Source

The rich phenol-contaminating POME was collected from an oil palm plantation in Trang Province of southern Thailand in June 2016. The dark wastewater was collected in a 50 mL sterile plastic bottle then transferred to our laboratory and stored at 4±1ºC until required for use.

POME Preparation

The POME was filled through a 0.25 μm Whatman paper filter to remove the suspended particles and contaminating microorganisms. The filtered POME was then placed in 250 mL Erlenmeyer flasks for further study.

Monitoring Culture Growing in POME

The cultures were transferred into 250 mL sterile Erlenmeyer flasks containing 90 mL of filtered POME and sterilized at 121±1ºC for 15 min. The POME was inoculated with 10 mL of consortium. After that, growth and ligninolytic activity were monitored. The cultures were grown at 30±1ºC with shaking at 150 rpm for five days, then the samples were collected every 24 h for five days. Yeast cell growth performance was determined by spectrophotometry at 600 nm. Growth curves were plotted as absorbance vs. time to monitor enzyme activity

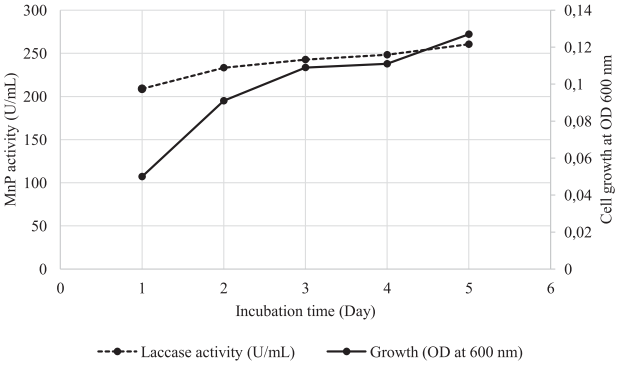

Fig. 2. Growing of *Galactomyces reessii* in raw POME and its manganese peroxidase (MnP) activity (U/mL).

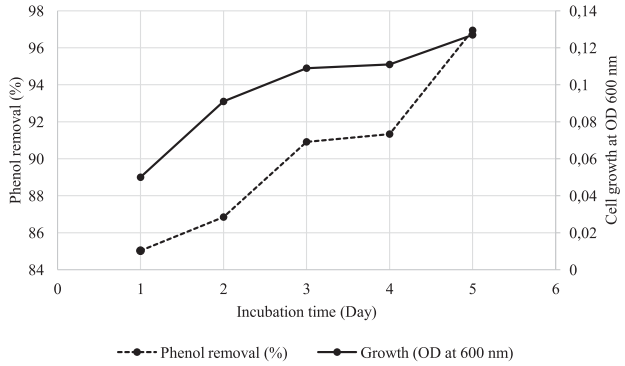

Fig. 3. Growing of *Galactomyces reessii* in raw POME and phenol removal (%).

and decreasing total phenol contamination. The cultures were centrifuged at 9,000 g for 10 mins to remove the yeast cells. The supernatants were filtered through filter paper and stored at 4±1ºC for further study. Determining total phenol content was performed following the Folin-Ciocalteu method. In short, 0.5 mL of POME was diluted with 4.5 mL of distilled water, and 5 mL of Folin-Ciocalteu reagent was added. After five minutes, 5 mL of 10% sodium carbonate (Na_2CO_3) solution was added and the mixture was briefly incubated and then cooled. The absorbance of the reaction was monitored at 760 nm. Total phenol was calculated by gallic acid equivalent [4]. Laccase (Lac) activity was determined at 420 nm using ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as a substrate. The assay mixture contained 1 mM ABTS in 100 mM sodium acetate buffer (pH 3.6) mixed with 10 µL aliquots of crude enzyme in a total volume of 200 µL [1]. Manganese peroxidase (MnP) activity was estimated by spectrophotometry at 415 nm using ABTS as a substrate. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmole of oxidized ABTS per minute.

Experimental Design

Optimizing phenol removal in raw POME was performed by *G. reessii*. The experimental design followed the Box-Behnken method. Cultural conditions were screened for four factors as incubation time (day),

Table 1. Factors and levels of each factor used for phenol removal.

Factor	Level		
	-1	0	1
Incubation times (days)	4	7	10
POME concentrations (%)	80	90	100
Culture concentration (%)	10	20	30
$CaCO_3$	1	5	10

Table 2. Box-Benhken design for phenol removal by *Galactomyces reessii*.

Run order	Incubation times (days)	POME concentrations (%)	Culture concentrations (%)	$CaCO_3$ (%)
1	4	100	20	5
2	7	90	20	5
3	7	90	30	10
4	10	90	30	5
5	4	80	20	5
6	7	90	10	10
7	4	90	10	5
8	4	90	20	10
9	7	80	30	5
10	4	90	30	5
11	7	90	20	5
12	7	90	10	1
13	7	100	30	5
14	7	100	20	1
15	7	80	10	5
16	7	100	20	10
17	10	90	20	1
18	10	100	20	5
19	7	100	10	5
20	10	80	20	5
21	4	90	20	1
22	7	80	20	1
23	7	90	30	1
24	7	90	20	5
25	7	80	20	10
26	10	90	10	5
27	10	90	20	10

POME concentration (%), culture concentration (%), and $CaCO_3$ (%). The filtered raw POME was used as the wastewater sample. All treatments were carried out in 250 mL Erlenmeyer flasks covered with sterile cotton and aluminium foil under aerobic conditions at 30±1ºC with shaking.

Statistical Analysis

Statistical data were performed by regression and ANOVA analysis at a 95% confidence level using Minitab® version 17.00 for Windows (Minitab, Pennsylvania, USA).

Table 3. Phenol removal in raw POME by *Galactomyces reessii*.

Treatment	Incubation times (days)	POME concentrations (%)	Culture concentrations (%)	$CaCO_3$ (%)	Phenol removal (%)
1	4	100	20	5	84.460
2	7	90	20	5	81.891
3	7	90	30	10	79.051
4	10	90	30	5	83.442
5	4	80	20	5	66.524
6	7	90	10	10	85.303
7	4	90	10	5	76.850
8	4	90	20	10	82.666
9	7	80	30	5	80.405
10	4	90	30	5	84.683
11	7	90	20	5	82.098
12	7	90	10	1	83.597
13	7	100	30	5	88.669
14	7	100	20	1	83.057
15	7	80	10	5	81.251
16	7	100	20	10	85.723
17	10	90	20	1	87.940
18	10	100	20	5	85.302
19	7	100	10	5	85.303
20	10	80	20	5	79.389
21	4	90	20	1	82.977
22	7	80	20	1	79.220
23	7	90	30	1	87.474
24	7	90	20	5	85.822
25	7	80	20	10	82.944
26	10	90	10	5	83.132
27	10	90	20	10	80.913

Results and Discussion

Growth in Raw POME

The analysis of autoclaved POME showed total phenolic compounds at 0.832 mg/mL. Growth ability, ligninolytic enzyme production, and phenol removal were monitored by spectrophotometry. After inoculation in POME, the culture was monitored for growth potential in the toxic wastewater. For ligninolytic enzyme production, data showed that *G. reessii* yeast produced laccase at 200.683, 221.843, 234.812, 249.147, and 314.676 U/mL after incubation at 30±1ºC under aerobic conditions with shaking for 1, 2, 3, 4, and 5 days of incubation, respectively (Fig. 1). Moreover, *G. reessii* secreted manganese peroxidase at 208.889, 223.333, 242.778, 248.333, and 260.556 U/mL over 1-5 days (Fig. 2).

Contaminated polyphenol content decreased with ligninolytic enzyme activity of the culture at 85.023, 86.847, 90.915, 91.336, and 96.947% after incubation under the same conditions (Fig. 3). On the other hand, Neoh et al. 2013 [10] used the filamentous fungi *Aspergillus fumigatus* isolated from POME sludge for the POME treatment, but they found the *A. fumigatus* successfully grown in POME supplemented with 5.7 g/L of glucose. Kietwanboot et al. 2015 [8] have been reported about the phenolics and colour removal activities in the white rot fungi *Trametes hirsute* strain AK04 that was found to be more tolerant of high POME concentration.

Table 4. Multiple regressions and significant terms ($p < 0.05$).

Source	DF	SS	MS	F-Value	P-Value	Remark
Regression	4	202.010984	50.502746	4.03	0.0133	Significant
$CaCO_3$	1	3.589428	3.589428	0.29	0.5977	-----
Culture (%)	1	5.724245	5.724245	0.46	0.5060	-----
POME (%)	1	152.517830	152.517830	12.18	0.0021	Significant
Times (Days)	1	40.179480	40.179480	3.21	0.0870	-----
Residual error	22	275.467641	12.521256	-----	-----	-----
Lack-of-fit	20	265.679712	13.283986	2.71	0.3036	-----
Pure error	2	9.787929	4.893964	-----	-----	-----
Total	26	477.478625	-----	-----	-----	-----

This system improved the fungal dephenolization and decolorization up to 82.2±3.8% and 87.1±1.1% after eight days of incubation. Moreover, Elmi et al. 2015 [6] applied microbial fuel cell (MFC) technology for POME treatment. The bacteria *Pseudomonas aeruginosa* NCIM 5223 was cultured in MFC anode under anaerobic conditions. The results showed 58% of chemical oxygen demand (COD) removal and 60% of color removal over eight days, while Othman et al. 2014 [13] treated the high phenol-contaminated POME by chemical method. In their work, the 10 g/L of activated carbon and 0.6 g/L of polyaluminium chloride were applied into the POME for wastewater treatment. Thus, *G. reessii* in our work showed high ability and efficiency for POME treatment, but needed low cost per volume of wastewater.

Optimization of Phenol Removal

The experimental design followed the Box-Behnken method. Cultural conditions were screened for four factors, including incubation time (day), POME concentration (%), culture concentration (%), and $CaCO_3$ (%) at three levels for each factor (Table 1) in 27 experiments (Table 2). Maximal phenol removal was determined at 100% POME concentration, 30% culture concentration, 5% $CaCo_3$, and seven days incubation time, when the culture removed 88.669% of the phenolic compounds (Table 3). Optimal treatment conditions regarding culture growth and ligninolytic enzyme production were determined at 228.333 U/mL manganese peroxidase and 255.973 U/mL laccase.

Statistical Analysis

Regression analysis was assessed using Minitab version 17.00 for Windows (Minitab, Pennsylvania, USA). Statistical analysis of the Box-Behnken design demonstrated regression values of $p < 0.05$, indicating that the model was significant (Table 4). Regression analysis was performed on the results. A first-order polynomial equation was derived representing phenol removal as a function of the independent variables. The regression equation of phenol removal was represented as:

$$\text{Phenol removal (\%)} = 45.492 - 0.1211CaCO_3\ (\%) + 0.0691\text{Culture (\%)} + 0.3565\text{POME (\%)} + 0.6099\text{Time (Days)}$$

Conclusions

This study revealed significant knowledge regarding the extracellular ligninolytic activity of termite-associated yeasts and their roles in phenol removal from POME. Results indicated that *G. reessii* had potential as an efficient strain for phenol removal in POME based on extracellular enzymes, including laccase and manganese peroxidase. Research regarding optimal conditions indicated seven days of incubation time, 30% culture concentration, and 5% $CaCO_3$ as the most suitable for contaminated phenol treatment in non-diluted raw POME. Knowledge gained from these studies can be utilised for the development of cost-effective wastewater treatment systems for effective phenol removal from POME.

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