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The culture or co-culture of *Candida rugosa* and *Yarrowia lipolytica* strain rM-4A, or incubation with their crude extracellular lipase and laccase preparations, for the biodegradation of palm oil mill wastewater

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ABSTRACT

Wastewater generated by the palm oil industry contains a high concentration of oil, grease and phenolic compounds, which makes it difficult to treat. The aim of this research was to evaluate the ability of free *Candida rugosa* and *Yarrowia lipolytica* rM-4A yeast cell cultures, as a single yeast strain and as a co-culture, as well as their crude extracellular enzyme preparations, to reduce the total phenolic compounds, triglycerides and chemical oxygen demand (COD) level in palm oil mill effluent (POME) that contains high amounts total phenolic compounds (9783 mg L^{-1}) and oil and grease (7762 mg L^{-1}). Culture with *Y. lipolytica* rM-4A effectively reduced the total phenolic content in undiluted POME, resulting in removal of 36% of the total phenolic content after 96 h. The highest removal of triglyceride (98.5%) and COD (60.3%) were observed in undiluted POME treated by the co-culture of *C. rugosa* and *Y. lipolytica* for 120 h. Triglyceride was removed rapidly by the extracellular lipase produced by *C. rugosa*, reaching 93% degradation after 48 h. This research revealed the excellent potential of both crude extracellular enzyme preparations and the co-culture of the two yeasts in the pretreatment of high fat and oil containing POME.

1. Introduction

Wastewater from oil mills usually contains a high concentration of oil and grease and has a high chemical oxygen demand (COD), biological oxygen demand (BOD) and level of phenolic compounds (Kanmani et al., 2015; Muktadirul Bari Chowdhury et al., 2013). The phenolic compounds lead to some biological effects such as phototoxic and antimicrobial effects (Ben Sassi et al., 2007). Oil and grease can form a film on a water surface that decreases the dissolved oxygen levels in the water and so, retards biological degradation (Rosa et al., 2009). Moreover, oil and grease may solidify at low temperatures, leading to several operational problems such as clogging of sewer systems, unpleasant odors and unsightly foam (Valladão et al., 2007). Accordingly, the removal of fat from this wastewater is important for the efficient operation of any biological treatment system. Although fungi, such as *Trametes versicolor* and *Funalia trogii*, have been recorded as potential bioremedial species for the removal of phenolic compounds (Ergül et al., 2010), their use at a large scale is difficult. Recently, the alternative use of yeast cells and their enzymes has gained more attention due to their ability to reduce the COD level and the ability of yeast to grow in high concentrations of phenolic compounds and a low pH (Ben Sassi et al., 2007). The yeasts *Yarrowia lipolytica* and *Candida* sp. have been reported as good candidates for wastewater purification (Gonçalves et al., 2009; Lanciotti, 2005), while the pretreatment of wastewater with lipase to hydrolyze and dissolve lipid content in wastewater has been reported to enhance the efficiency of anaerobic digestion

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and biogas production (Mendes et al., 2006; Mobarak-Qamsari et al., 2012).

Lipases, or triacylglycerol acylhydrolases (EC 3.1.1.3), are one of the most important enzyme types that catalyze the hydrolysis of the carboxyl esters of triglycerides to diglycerides, free fatty acids and glycerol. Furthermore, lipases also catalyze several biotransformations such as esterification and transesterification. This ability has made them more and more popular in the food, cosmetic, organic synthesis, detergent and pharmaceutical industries as well as in wastewater treatment (Salihu et al., 2011). Lipases can be found in microorganisms, plants and animals, but those from microbes have mostly been used due to their stability in organic solvents, ease of cultivation, lack of required cofactors, broad substrate specificity and ability to be produced in high yields (Kumar et al., 2012). Among the organisms that produce lipase, the yeast *Candida rugosa* has been reported as a strong lipase producer (Mobarak-Qamsari et al., 2012; Morais Júnior et al., 2016).

Laccases, or benzenediol oxygen oxidoreductases (EC 1.10.3.2), catalyze the oxidation of many compounds, including mono-, di-, poly- and amino-phenols. When compared with other oxidizing enzymes that usually need additives to be catalytically active, laccase needs only oxygen for catalyzing the reaction (Li et al., 2009). Laccases have been used in many industrial applications, including biodegradation of xenobiotic compounds, detoxification of industrial wastewater and synthetic dye decolorization (Theerachat et al., 2012).

The aim of this study was to determine the potential of crude extracellular enzyme preparations and free yeast cell cultivation, both as single yeast strain cultures and co-cultures of *C. rugosa* and *Y. lipolytica* rM-4A, in the pretreatment of palm oil mill effluent (POME) with a high oil concentration. The efficiency in reducing the total phenolic compounds, COD and triglyceride levels, were determined and compared. The laccase gene (*lcc1*) from the fungus *Trametes versicolor* was previously engineered and cloned into the genome of *Y. lipolytica* JMY1212, and the rM-4A strain showed the highest laccase activity (Theerachat et al., 2012) and so was used in this study as a source of laccase.

2. Materials and methods

2.1. Microorganism

The *C. rugosa* CU1 isolate and *Y. lipolytica* strain rM-4A used in this study were provided by Biofuels by Biocatalysts Research Unit, Thailand. The yeast *C. rugosa* was isolated from soil samples collected in Thailand. A partial 26S ribosomal RNA sequence was submitted in GenBank with accession number KY563206. The yeast was cultivated in YM medium (yeast extract, 3.0 g L^{-1}; Bacto-peptone, 5.0 g L^{-1}; malt extract, 3.0 g L^{-1}; D-glucose, 10.0 g L^{-1}) and on YM agar (YM plus 20.0 g L^{-1} agar). The *Y. lipolytica* strain rM-4A was engineered by insertion of the mutant laccase gene into genome of *Y. lipolytica* strain JMY1212 as previously reported (Theerachat et al., 2012) and was grown in YPD medium (yeast extract, 10.0 g L^{-1}; Bacto-peptone, 10.0 g L^{-1}; glucose, 10.0 g L^{-1}) and YPD agar (YPD plus 15.0 g L^{-1} agar). Both yeasts were grown at 30 °C for 48 h on agar medium and subsequently maintained at 4 °C for storage.

2.2. Collection and characterization of the POME (wastewater)

The POME used in this study was collected from a palm oil mill in Chonburi province, Thailand, and kept at −20 °C until used. This POME was mainly produced by the palm oil production process. The sample was then characterized for its pH and level of total solids (TS), total suspended solids (TSS), volatile solids (VS), oil and grease, BOD, COD, total Kjeldahl nitrogen, total phosphorus, copper, calcium, magnesium, total phenolic content and reducing sugar, as outlined in section 2.8.

2.3. Extracellular lipase production

A single colony of *C. rugosa* was pre-grown in YM broth at 30 °C, 200 rpm for 24 h. This culture was then used to inoculate 25 mL of production medium (yeast extract,15.0 g L^{-1}; $MgSO_4x7H_2O$, 1.0 g L^{-1}; KH_2PO_4, 10.0 g L^{-1}; cotton seed oil,10.0 g L^{-1}; pH = 7.0) in a 250-mL Erlenmeyer flask to an initial optical density (OD_{600}) of 0.1. It was then incubated at 30 °C, 200 rpm for 120 h. The culture broth was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was harvested and filtered through a 0.2 micron filter to remove the cells. This sterile filtered culture supernatant was used as the source of extracellular lipase.

2.4. Extracellular laccase production

A single colony of *Y. lipolytica* rM-4A was pre-grown in YPD broth at 28 °C, 135 rpm for 24 h. The culture was then used to inoculate fresh YTD medium as described previously (Theerachat et al., 2012). The culture supernatant was harvested and sterile filtered as in section 2.3 and used as the source of extracellular laccase.

2.5. Enzyme assay

Lipase activity was determined spectrophotometrically using *p*-nitrophenyl butyrate (*p*-NPB) (Sigma, USA) as the substrate as previously reported (Piamtongkam et al., 2011). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1μmole of butyric acid from *p*-NPB per minute at 37 °C, pH = 7.2.

Laccase activity was measured using 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma, USA) as the substrate as previously described (Theerachat et al., 2012). All enzyme assays were carried out in three replicates.

2.6. POME pretreatment by crude enzymes

2.6.1. COD removal in non-sterile condition

The POME used in this study, which contained 2762 mg L^{-1} of oil and grease (Table 1), was centrifuged at 4000 rpm for 15 min to eliminate solids and then diluted two-fold in water. To stimulate a

Table 1
Initial characteristics of the POME used in this study.

Parameter (mg L^{-1})	Value
pH	4.3
TS	56,100
TSS	12,430
VS	46,930
Oil and Grease	2762
BOD	33,090
COD	34584
TKN	1693
Total phosphorus	44.3
Copper	0.72
Calcium	86.4
Magnesium	269
Total phenolic content	9782.5
Reducing sugar	3449.8

TS, total solids; TSS, total suspended solids; VS, volatile solids; BOD, biochemical oxygen demand; COD, chemical oxygen demand; TKN, total Kjeldahl Nitrogen.