

Isolation and Molecular Characterization of Microorganisms with Potential for the Degradation of Oil and Grease from Palm Oil Refinery Wastes

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In this study we isolated and characterized native microorganisms with the ability to degrade oil and grease (O&G) and evaluated their potential for the treatment of POMEs from a palm oil refining process. Yeast and bacterial isolates were obtained from solid and liquid wastes from a grease trap of a palm oil refining process, based on their ability to use palm oil as sole carbon source in solid medium. Molecular identification of microorganisms was performed by PCR techniques, revealing that isolates corresponded to *Candida* and *Bacillus* species, with a high degree of similarity with reported O&G-degrading organisms. Five out of these isolates showed lipolytic activity evidenced by changes in the turbidity, colour and produced a substantial decrease in O&G concentrations in liquid MBS cultures containing palm oil. These isolates promoted the highest O&G decrease in POME samples with 56 %, 77 %, 78 %, 76 % and 79 % O&G removal after 72 hours respectively. A microbial consortia composed of five degrading yeasts produced a O&G reduction up to 84 % in POME samples after 48 hours, evidencing a synergic effect of the microorganisms. The results of this study showed that bioaugmentation of polluted wastewaters from palm oil extraction with native microorganisms isolated from oily residues can be efficiently used to greatly improve the removal of grease, oils and organic matter.

1. Introduction

The palm oil industry is currently a world leader in the supply of oils and fats and one of the sectors of the highest economic importance because of the versatility of applications of their by-products, such as cooking oil, special fats, margarines, soaps, detergents, cosmetics, toothpastes, candles, lubricants, biofuels and electric power, among many others. Currently there are about five million hectares of palm planted in the world, representing 16 million tons of annual production. Colombia is the first producer of palm oil in America and the fourth largest in the world after Malaysia, Indonesia and Nigeria (USDA 2015). Much of this oil is obtained from the African oil palm (*Elaeis guineensis* Jacq.) and hybrids with other species as well. However, the improper disposal of wastes from oil refining -containing oils, fats, polluted effluents and sludges- causes a deterioration on the environment and human health because of their carcinogenic, toxic and polluting effects. One of the main wastes derived from palm oil processing are the palm oil mill effluents (POMEs), an oily wastewater generated from milling activities. POME composition include high amounts of oil and grease (O&G), total suspended solids (TSS), chemical oxygen (COD) and biochemical oxygen demand (BOD) which counts for the majority of the contaminant effects on watercourses due to their highly polluting properties and acidic nature. The uncontrolled disposal of these wastes into water bodies may produce important effects such as an alteration of pH, an increase in the organic matter, BOD, COD, and prevention of the passage of light and oxygen generating eutrophication and potentially, toxic compounds (Rupani et al. 2010). Thus, treatment of POMEs is important to avoid environmental pollution of water bodies.

Bioremediation of POMEs has been demonstrated to be an efficient method for the degradation of organic pollutants, enhancing the overall degradative performance by using microorganisms with high degradation ability of specific environmental pollutants (Ojonoma and Udeme, 2014). However, while several microbial species with the ability to remediate POMEs such as *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Candida*, *Saccharomyces*, *Pichia* and *Yarrowia* have been identified (Vijayaraghavan et al. 2007), there are only few studies on the degradation of these wastewaters using native aerobic microbial consortia consisting of microorganisms isolated from highly polluted wastes. Moreover, the use of native microorganisms for the remediation of POMEs would improve the adaptation, survival and degrading ability of microorganisms on effluents containing high amounts of toxic contaminants. Thus, the aim of this study was to isolate and characterize native microorganisms with the ability to degrade oil and grease (O&G) and evaluate their potential for the treatment of POMEs from a palm oil refining process.

2. Materials and Methods

2.1 O&G derived wastes

POME samples obtained from a grease trap of a palm oil refining process were used in this study. Composite samples of two liters were obtained in a simple random strategy at different points and depths across the oil trap. A physicochemical characterization consisting in the measurement of pH, COD, TSS and O&G content was conducted for all samples (Table 1).

2.2 Microbial isolation

Microbial isolation from oily wastes was performed by diluting 10 gr (for solid samples) or 10 ml (for liquid samples) in 70 ml of Basal Saline Medium (BSM) containing (g l^{-1}): $(\text{NH}_4)_2\text{SO}_4$, 3; K_2HPO_4 , 0.9; KH_2PO_4 , 0.6; MgSO_4 , 0.2; CaCO_3 , 0.5; yeast extract, 0.1. Flasks were incubated with constant agitation at 30 °C for 48 hours and then 10 ml were diluted in 90 ml of BSM using 0.05 % palm oil as sole carbon source. One ml of supernatant was used to inoculate plates of solid BSM and after 96 h of incubation at 30 °C. Individual colonies were picked and transferred to new plates of BSM containing palm oil as carbon source.

2.3 Molecular identification and characterization

Genomic DNA extraction from yeast and bacterial isolates was performed by using the Wizard Genomic DNA Purification Kit (Promega, USA). Bacterial Isolates were identified by the amplification and sequencing of the 16S rRNA gene using the universal primers P27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane 1991), and yeast isolates were identified by sequencing the ITS1, ITS2 and 5.8S rRNA regions using the primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) using conditions described by White et al. (1990). PCR products were analyzed by agarose gel electrophoresis and purified using the Column-pure DNA Gel Recovery Kit (abm, Canada). DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen, USA), and an Applied Biosystems ABI 3100 genetic analyzer using oligonucleotides P27F and ITS5 as sequencing primers. BLAST (Altschul et al. 1990) was used for homology searching and for the identification of microorganisms. Phylogenetic analysis was performed using the MEGA 6 software package (Tamura et al. 2013).

Table 1. Physicochemical characterization of four POME samples (mean) from a grease trap of a palm oil refining process. COD: Chemical Oxygen Demand; BOD: Biological Oxygen Demand; TSS: Total Suspended Solids; O&G, Oil and Grease.

Parameter	Mean (SD)
pH (at 25°C)	5.43 (± 0.51)
Temperature	30.4 (± 1.44)
COD (mg L^{-1})	11,247 (± 1.85)
BOD (mg L^{-1})	5,925 (± 1.86)
TSS (mg L^{-1})	2,943 (± 0.76)
O&G (mg L^{-1})	4,907 (± 0.67)

2.4 Evaluation of O&G degradation by single microorganisms and a microbial consortium

O&G-degrading potential of isolated microorganisms was assessed in BSM tubes containing 2,000 mg L^{-1} palm oil. For this, 20 ml of 10^9 colony forming units (CFU) ml^{-1} lag-phase cultures from each isolate were inoculated into glass flasks containing 200 ml BSM plus palm oil and incubated at 27 ± 3 °C with constant

aeration. The degrading ability of individual isolates was determined by the decrease in O&G content (measured as COD) during 48 and 72 hours with respect to controls. Subsequently, to test for possible synergistic effects between isolates, consortia were constructed using combinations of yeasts and bacteria. The construction of a mixed inoculum with lipolytic activity was performed by selecting bacterial and yeast isolates based on their ability to use palm oil, reported capacity to metabolize O&G and the lack of antagonism between them. Removal ability of O&G was evaluated in a four-liter bioreactor using POMEs from the palm oil refining process. Each microbial isolate was grown on tubes containing liquid BSM plus oil palm, inoculated in the bioreactor at final concentration of 10^9 CFU each (1:1 ratio) and the volume adjusted to four liters with POME. The process was carried out for 48 h at 27 ± 3 °C with constant aeration. The degradation of O&G was indirectly measured by means of the COD removal, as it allows the quantitation of organic matter in liquid samples (including O&G). O&G removal activity was also determined by means of the O&G disappearance according to the ASTM Soxhlet method D5369 at the end of the process. COD values were determined according to method HACH 8000 (HACH, 2003). All assays were carried out in triplicate.

2.5 Statistical Analysis

Data from COD measurements were analyzed by analysis of variance (ANOVA) followed by a Bonferroni multiple comparison test with SPSS Statistics Software version 19 (IBM), considering statistically significant differences those with a p value < 0.05. Molecular weights of amplified bands were calculated using Photo-CaptMw v10.01 software (Vilber Loumat, France).

3. Results and Discussion

Bioremediation is a good alternative for the management of POMEs and other hazardous wastes from palm oil refining. Studies have shown the degradation of high amounts of greases and other organic contaminants present in POMEs by aerobic bacteria such as *Pseudomonas* and *Bacillus* species, as well as yeasts such as *Candida*, *Saccharomyces*, *Pichia* and *Yarrowia* species isolated from the same source of contamination (Vijayaraghavan et al. 2007). In this study a total of 20 microbial isolates, corresponding to 10 yeast and 10 bacteria, were obtained from liquid and solid samples based on their ability to use palm oil as sole carbon source in solid medium. Only 10 out of these 31 microbial isolates showed visible lipolytic activity evidenced by a reduction in the palm oil layer present in MBS tubes. Further molecular characterization of ribosomal sequences revealed that yeast isolates corresponded to *Candida palmioleophila* and bacterial isolates to *Bacillus* sp. (Table 2). To determine the phylogenetic position of the microbial isolates, ITS1, ITS2 and 5.8S rRNA and 16S rRNA sequences were aligned with the corresponding sequences of several known O&G-degrading organisms. The ITS-based phylogenetic tree showed that yeast isolates are closely related between them and with these reported O&G-degrading yeasts (Figure 1). These microorganisms have been previously described to degrade vegetable, animal and mineral O&G, mainly due to the production of lipases and other enzymes capable of completely or partially metabolize these compounds (Lotti and Alberghina, 2007). *Candida palmioleophila* (formerly known as *Toluroopsis candida*) is a yeast reported as capable to decolorate azo dyes (Jafari et al. 2013) and assimilating crude palm oil (Nakase et al. 1988). In spite of its potential to degrade oils, to our knowledge, there are no reports of the use of *Candida palmioleophila* for the biological treatment of POMEs.

Table 2: Molecular identification of fungal and bacterial native isolates from palm oil refinery wastes

Isolate	Closest related species (GenBank accession number)	Identity	Phylum	Class
SACL01	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL03	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL04	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL05	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL06	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL08	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL09	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACL11	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACB02	<i>Candida palmioleophila</i> (KJ705005.1)	100 %	Ascomycota	Saccharomycetes
SACB10	<i>Bacillus</i> sp. (KR262848.1)	99 %	Firmicutes	Bacilli
SACB10	<i>Bacillus</i> sp. (KR262848.1)	99 %	Firmicutes	Bacilli

72 % O&G degradation (809 mg L^{-1}) after 48 hours. Comparatively, a non-inoculated control showed a COD reduction of 51 % ($1,600 \text{ mg L}^{-1}$) and 50 % O&G degradation (809 L^{-1}), and thus inoculation with the constructed community described here represented respective increases for COD reduction and O&G removal of 225 % and 144 %. These removal levels in control could be attributed to native microorganisms already present in palm oil-wastewaters, which in fact contains most of the isolated microorganisms described in this study. Notably, the results from O&G quantitation using COD during POME treatment corresponded with those obtained with the Soxhlet method (51 vs 50 % and 75 vs 72 %), evidencing a good correlation between the two methods.

Overall, O&G removal by the constructed consortia described here was shown to be higher and faster than with other reported mixed inocula. For example, a mixed inocula composed by one strain of *Candida cylindracea* and three of *Yarrowia lipolytica* decreased 30-70 % COD after 100 hours of treatment (Gonçalves et al., 2009). Additionally, the results presented here are consistent with previous reports showing an enhanced degradation in POME by bacteria-yeast co-cultures, reaching up to 72 % O&G and 80 % COD reduction (Bhumibhamon et al., 2002). This highlights the advantage of using mixed cultures for the bioremediation of POMEs, which could confer several advantages such as an increased co-metabolism and improved tolerance to pH and temperature variations (Boopathy, 2000). Since most of the microbial lipases are inducible and secreted as extracellular enzymes upon induction with fatty acids (Lanka et al., 2015), the use of yeast-bacteria mixed inocula would also improve the degradation of high molecular weight greases by yeast after the induction of the bacterial enzymes able to degrade low molecular weight molecules (Bala et al., 2014). Also, this type of metabolism could be effectively used for the degradation of other related pollutants. In fact, an increased co-metabolic degradation of total petroleum hydrocarbons in soil has been achieved by the same microorganisms able to remediate POMEs (Hamzah et al., 2014).

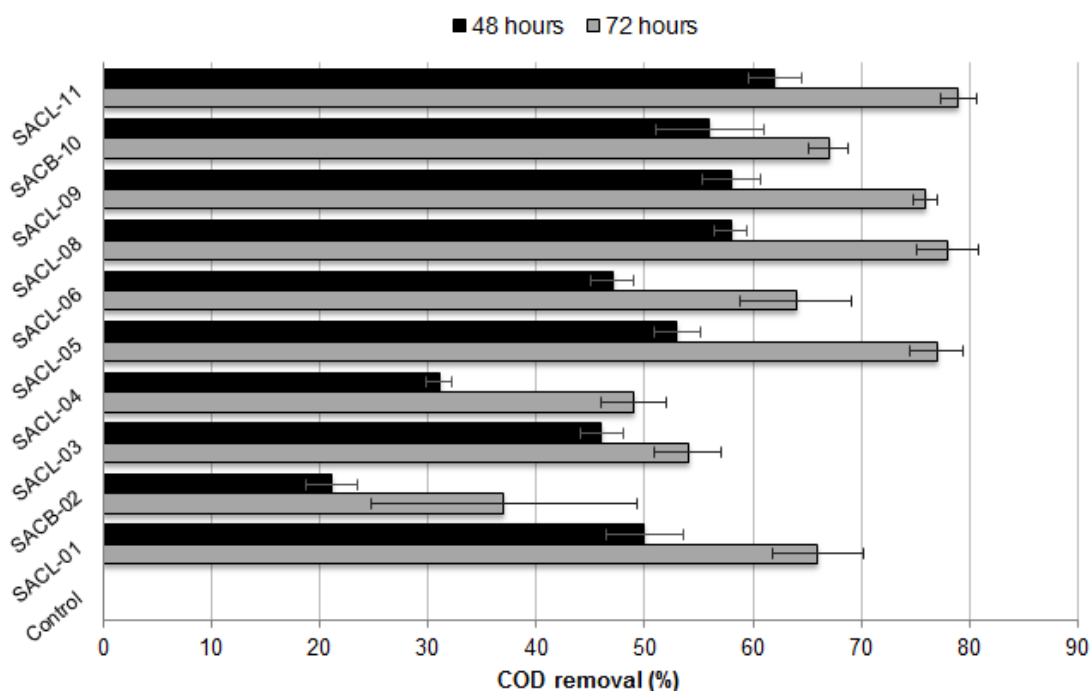


Figure 1. O&G removal (as COD) by single microbial isolates in liquid MBS medium with 0.05% palm oil.

4. Conclusions

The results of this study showed that the bioaugmentation using native microorganisms isolated from oily residues, either single or mixed, can be efficiently used to greatly improve the removal of grease, oils and organic matter present in wastewaters from palm oil extraction. Our results demonstrated the ability of these isolates to use palm oil as sole carbon source and effectively decrease the concentration of organic compounds in POME, including grease and oil, in a short period of time (48 hours). The use of these

microorganisms may provide adaptive advantages that could improve POME remediation process, especially when lipolytic mixtures of native bacteria and yeast are used. Further studies would be necessary in order to clarify the role of each isolate in the mixed inoculum during the degradation of O&G in POMEs, as well as to test the potential to produce and secrete lipolytic enzymes and other oxidoreductases.

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