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Extracellular laccase production and phenolic degradation by an olive mill wastewater isolate

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SUMMARY: Olive mill wastewater (OMWW) presents a challenge to the control of effluents due to the presence of a high organic load, antimicrobial agents (monomeric-polymeric phenols, volatile acids, polyalcohols, and tannins), salinity and acidity. In this study, the production of extracellular laccase, monomeric or polymeric phenol, from an OMWW isolate based on its ability to biodegrade phenols and gallic acid as a model of phenolic compounds in OMWW was investigated. Phylogenetic analysis of the 16S RNA gene sequences identified the bacterial isolate (*Acinetobacter* REY) as being closest to *Acinetobacter pittii.* This isolate exhibited a constitutive production of extracellular laccase with an activity of 1.5 and 1.3 U ml/L when supplemented with the inducers $CuSO_4$ and $CuSO_4$+phenols, respectively. Batch experiments containing minimal media supplemented with phenols or gallic acid as the sole carbon and energy source were performed in order to characterize their phenolic biodegradability. *Acinetobacter* REY was capable of biodegrading up to 200 mg/L of phenols and gallic acid both after 10 h and 72 h, respectively.

KEYWORDS*:* Acinetobacter *REY; Biodegradation; Extracellular laccase; Olive mill waste water; Phenolic compounds*

RESUMEN: ***Producción de lacasa extracelular y degradación de compuestos fenólicos mediante un aislado de aguas residuales de almazara***. Las aguas residuales de almazara (OMWW) presentan un desafío a los efluentes debido a la presencia de una carga orgánica alta, agentes antimicrobianos (fenoles monoméricos y poliméricos, ácidos volátiles, polialcoholes y taninos), salinidad y acidez. En este estudio, se investigó la producción de lacasa extracelular a partir de un aislado de OMWW basado en su capacidad para biodegradar fenol y ácido gálico como modelo de compuestos fenólicos en OMWW. El análisis filogenético de las secuencias del gen de ARN 16S identifico el aislado bacteriano (*Acinetobacter* REY) como el más cercano a *Acinetobacter pittii*. Este aislado exhibió producción constitutiva de lacasa extracelular con una actividad de 1.5 y 1.3 U mL/L cuando se suplemento con los inductores CuSO4 y CuSO4 + fenol, respectivamente. Se realizaron experimentos en lotes que contenían medios mínimos suplementados con fenol o acido gálico como la única fuente de carbono y energía con el fin de caracterizar su biodegradabilidad fenólica. *Acinetobacter* REY fue capaz de biodegradar hasta 200 mg/L de fenol y acido gálico después de 10 y 72 h, respectivamente.

PALABRAS CLAVE**:** Acinetobacter *REY; Aguas residuales de almazara; Biodegradación; Compuestos fenólicos; Lacasa de células extracelulares*

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1. INTRODUCTION

Approximately 5.4×10^6 tons of olive mill wastewater (OMWW) are produced annually worldwide. OMWW is a recalcitrant waste that has high phenolic, lipid, organic acid and tannin contents. The phenolics are present as a mono/polymeric aromatic mix which gives the OMWW its brownish color. Uncontrolled disposal of OMWW is becoming a serious environmental problem, due to its high organic content which consists mainly of antimicrobial and phytotoxic phenolic compounds (Ruiz *et al*., 2002).

The importance of using enzymes in industrial and agricultural waste treatment has increased in recent years. Laccase (benzenediol: oxygen oxidoreductases, copper-containing polyphenol oxidase EC1.10.3.2) is one, inter alia, which is currently the focus of much attention because of its diverse applications. These include dye decolorization, pulp bleaching, waste detoxifications and bioremediation of environmental pollutants, synthesis of organics or electrocatalysis (Kunamneni *et al*., 2008). Laccases can catalyze a broad range of phenolic and nonphenolic compounds without any requirement other than oxygen. This makes them very useful in biotechnological applications. Laccases catalyze phenols such as *ortho*- and *para*-diphenols, aminophenols, polyphenols, methoxy-substituted phenols, 2,6-dimethoxyphenol, phenolic acids, polyamines, aromatic amines, phenylene diamine, lignins, aryl diamines, as well as several other nonphenolic compounds (hydroquinone, guaiacol) and some inorganic ions (Atalla *et al*., 2013).

Fungal laccases have been widely studied for the detoxification of pollutants in the environment, revaluation of industrial wastes and wastewater treatment (Bertand *et al*., 2013). However, little is known about the use of bacterial laccases for these applications. Recent studies showed the extracellular production of laccase by several bacterial strains, including *Pseudomonas putida* F6, *P. putida* MTCC 7525, *Pseudomonas* sp. LBC1, *P. putida* LUA15.1, *Escherichia coli*, *Bacillus halodurans*, *B. subtilis* SF, *Bacillus* sp. HR03, *Azospirillum lipoferum*, *P. desmolyticum* NCIM 2112, *B. pumilus* and *B. subtilis* WP1 (Kuddus *et al*., 2013; Muthukumar and Murugan, 2014; Verma *et al*., 2016). Most bacterial laccases are stable, highly thermo-tolerant and maintain their activity under neutral to alkaline conditions. They are less dependent on metal ions and less susceptible to inhibitory agents than fungal laccases (Baldrian and Šnajdr, 2006). Bacterial laccases therefore continue to draw attention as an alternative to chemical processes which would enable the industry to meet the increasingly stringent environmental requirements of reducing the environmental xenobiotic load. For example, several studies showed the use of laccase from different bacteria for detoxifying a wide range of pollutants. This has made them useful in many industries, including wine, paper, pulp and textile industries. Other applications, including biobleaching, enhanced saccharification and biocatalytic arylation, were also reported (Sondhi *et al*., 2015; Suljić *et al*., 2015). The use of laccase for treating OMWW may present a great opportunity for successful biological treatment by overcoming the presence of the major obstacle: phenolics. Eliminating or significantly reducing the major anti-microbial agent and oxygen consumer allows for a more cost-effective treatment solution for OMWW. It is therefore possible that the OMWW flora contains isolates that have already developed resistance to this virulent medium, and it may be possible to exploit their ability to produce the target enzyme, laccase.

Extraction of extracellular enzymes from industrial and agricultural waste isolates has great potential for reducing the pollutant load on the environment. The aim of the present study was therefore to isolate a bacterium from OMWW based on its ability to biodegrade phenols and gallic acid as a model for phenolic compound biodegradation as a pre-treatment or as an additive treatment for use in OMWW remediation. This isolate, which originated from an OMWW, was screened for the production of extracellular enzymes, including amylase, protease, lipase and laccase. Additional information on the potential of bacterial laccases for wastewater treatment is discussed.

2. MATERIALS AND METHODS

2.1. OMWW characterization and sampling

The OMWW used in this work was collected from Maghar, the Galilee region, Israel. The main properties of the OMWW used for bacterial isolation were determined as described in standard methods (Rice 2012) and are reported in Table 1. The phenolic content of the OMWW was characterized as described by Azaizeh *et al*., (2012) and is shown in Figure 1.

2.2. Isolation of phenolic-degrading bacteria and identification of the isolate by 16s rRNA analysis

Microorganisms were isolated by their ability to grow on phenols as the sole carbon source, as described in detail by Kurzbaum *et al*., (2010). After incubation at 37 °C for 24 h, the fastest growing colonies were isolated using several isolation cycles. The fastest growing colony was then identified by 16S rRNA analysis. In order to verify the phylogenetic affiliation of the selected isolate, a single colony was collected for DNA isolation and subjected to PCR analysis using primers targeting the 16S rRNA gene [27F (AGAGTTTGATCMTGGCTCAG) and

1513R (ACGGYTACCTTGTTACGACTT)], by the direct colony PCR method as previously described (Iasur-Kruh *et al*., 2011). PCR (25 µL) contained 10 µL of Apex™ Taq DNA Polymerase Master Mix (Genorama, Tartu, Estonia), 5 pmol of each primer, 12.5 µL DDW and 1 µL DNA template. The PCR procedure was as follows: DNA was denatured at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 sec each, 58 °C for 30 sec and 72 °C for 1 min, followed by 5 min at 72 °C. The PCR product was sequenced by Hy-labs (Rehovot, Israel). A neighbor-joining (NJ) tree based on nearly complete 16S rRNA gene sequences showed the phylogenetic position of the OMWW isolate to be a closely related species from the genus *Acinetobacter*. Multiple alignments were computed using MUSCLE (Edgar, 2004) and the NJ tree was calculated with Kimura 2 parameters model in MEGA7 (Kumar *et al*., 2016) with 1000 bootstrap replicates. *Moraxella lacunata* ATCC 17967 was used as an out group.

2.3. Sequence accession

The sequence of the bacterium isolated from the OMWW was deposited in GenBank, NCBI (KY828225).

TABLE 1. Different parameters of crude OMWW and s-TP fraction.

Parameter	Crude OMWW	Fraction s-TP
pH	4.64 ± 0.12	2.99 ± 0.05
Total COD	133519 ± 4512	3333 ± 663
TSS	6990 ± 180	129.7 ± 12.8
Total Phenol	3250 ± 180	157.9 ± 4.3
TKN	280.5 ± 22	1.12 ± 0.15

All units are expressed in mg/L, except pH.
± indicates standard deviation

2.4. Inoculum preparation for degradation studies

The selected pure colonies were aseptically transferred to 250 ml Erlenmeyer flasks containing a mineral salt medium (MSM) comprised of the following salts (g/L): $NaHPO_4 \cdot 12\ H_2O$-6.15 g/L, KH_2PO4-1.52 g/L, $(NH_4)_2SO_4$-1 g/L, $MgSO_4 \cdot 7H_2O$-0.2 g/L, and the trace metals: EDTA-0.5 g/L, $FeSO_4 \cdot 7H2O$-0.2 g/L, $ZnSO_4 \cdot 7H2O$-0.01 g/L, $MnCl_2 \cdot 4H_2O$-0.003 g/L, H_3BO_3-0.03 g/L, $CoCl_2 \cdot 6H_2O$-0.02 g/L, $CuCl_2 \cdot 2H_2O$-0.001 g/L, $NiCl_2 \cdot 6H_2O$-0.002 g/L, $NaMoO_4 \cdot 2H_2O$-0.003 g/L. The final pH of the medium was 7 (Muñoz *et al*., 2007). Phenols and gallic acid, 50 mg/L each as a carbon source, were added to the MSM for growth of the new isolate. The cultures were incubated in an orbital shaker at 37 °C and 100 rpm for 24 h.

2.5. Growth kinetics and phenol/gallic acid biodegradation

The bacterial strain isolated from the OMWW was evaluated for growth in the presence of either phenols or gallic acid (separately) as the sole carbon and energy source in order to characterize its potential for phenol/gallic acid biodegradation.

For the phenol biodegradation experiment, the isolate suspension was transferred to a set of sterile Erlenmeyer flasks with 100 mL MSM and different initial phenol (Alfa Aesar, UK) concentrations (30, 50, 100 and 200 mg/L). All flasks were supplemented with an initial equal biomass of ~0.144 CFU/mL (absorbance A_{600nm} 0.018). Sterile control flasks contained medium with the respective phenol concentration, without bacterial cells. All treatments and controls were performed in triplicate and incubated in an orbital shaker at 37 °C and 100 rpm (Lab centrifuge, MRC Ltd., Israel). In order to evaluate growth and degradation kinetics, the biomass growth was monitored by the

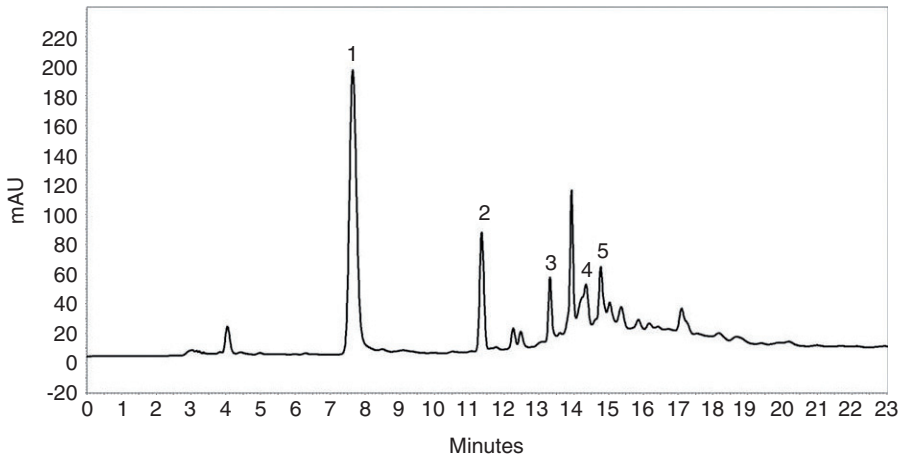

FIGURE 1. Chromatogram of the fresh s-TP fraction of OMWW at 280 nm. Hydroxtyrosol (1), tyrososl (2), vanillic acid (3), ferulic acid (4), and p-coumaric acid (5).

absorbance of the suspension at 600 nm (Ultrospec 2100 pro, Amersham Biosciences, USA) and phenol concentrations were determined at different time points using the direct spectrometric method with 4-aminoantipyrine (4-AAP) as a color reagent as described in detail in Rice *et al*., (2012). The method is based on a reaction between phenols and 4AAP in the presence of ferricyanide at pH 7.9. Absorbance was measured at 500 nm.

The experimental setup for the gallic acid biodegradation experiment was identical to the phenol treatments, but with initial gallic acid concentrations of 25, 50, 100 and 200 mg/L as the sole carbon source. Three replicates were performed for each gallic acid concentration and control. The samples were centrifuged and the supernatant was sampled at different time points for gallic acid measurement according to the Folin–Ciocalteau reagent (Sigma, Israel) method with gallic acid as the standard (10-100 mg/L) (Azaizeh *et al*., 2015). The degradation kinetics of gallic acid were calculated in terms of % reduction.

2.6. Screening for extracellular enzyme

Pure cultures isolated from the OMWW were inoculated on skim milk agar plates (Fulzele *et al*., 2011), tributyrin nutrient agar plates, starch agar plates (Mishra and Behera, 2008) and tryptone yeast broth (Dalfard *et al*., 2006) for screening protease, esterase, amylase and laccase, respectively.

2.7. Bacterial growth conditions for laccase production

The OMWW isolate (*Acinetobacter* REY) was inoculated into Erlenmeyer flasks containing 100 mL of sterile tryptone yeast broth (TYB) containing 0.15% yeast extract (RM027, Himedia), 0.15% tryptone (Tryptone type-1 RM014, Himedia) and 0.5% sodium chloride as described earlier by Dalfard *et al*., (2006). The bacterial biomass was measured after 0, 24, 48, 72 h under shaking (100 rpm) at 37 °C, as described above, and the culture supernatant was collected after centrifugation at 10000 rpm for 10 min at 4 °C for extracellular laccase assay (Verma *et al*., 2016). In order to characterize the effect of inducers on laccase production by the isolate, treatments containing 200 µmol/L $CuSO_4 \cdot 5H_20$ alone (Kuddus *et al*., 2013; Sondhi *et al*., 2015; Verma *et al*., 2016) and 200 µmol/L $CuSO_4 \cdot 5H_20$ with 50 mg/L phenols were performed. Sterile controls contained 100 mL of TYB (with and without inducer) without the addition of the isolate. All experiments were conducted in triplicate and the presented results are the average of three independent experiments.

2.8. Extracellular laccase activity

The enzyme assay was performed on the filtrate supernatant using 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) (Sigma, Israel) as the substrate for laccase activity, as described earlier (Tahmasbi *et al*., 2016), with some modifications. The per mL assay mixture consisted of 200 µL of crude extracellular filtrate (enzyme source) and 800 µL aliquots of 1 mmol/L ABTS prepared in a 0.1 mol/L sodium acetate buffer (pH 4.5) (Verma *et al*., 2016). The oxidation of ABTS was monitored by determining the increase in absorbance at 420 nm (ε420, 36000 L/(mol.cm) at a path length of 1 cm) after incubation at 32 °C for 10 min. Blank samples were identical, except for the addition of 200 µL of 0.1 mol/L sodium acetate buffer (pH 4.5) instead of the crude extracellular filtrate. All sampling and measurements were performed in triplicate. The change in absorbance in 10 min is equivalent to 1 µmol/L of ABTS oxidized in 10 min. The results were then converted to 1 µmol of ABTS oxidized per min in order to calculate the International Units (IU). 1U is the amount of enzyme required to oxidize 1 µmol of ABTS substrate per min. The laccase activity in U/mL was calculated by the formula:

$$EA = A \times V / t \times \varepsilon \times v$$

Where
EA = Enzyme activity (IU)
A = Absorbance at 420 nm
V = Total mixture volume (mL)
v = Enzyme volume/Bacterial culture filtrate (mL)
t = Incubation time (min)
ε = Extinction coefficient for ABTS (36000 L/(mol·cm))

3. RESULTS

3.1. Physico-chemical analysis of OMWW

The fresh OMWW was analyzed according to standard methods (Rice *et al*., 2012). The parameters are shown in Table 1. The phenolic profile HPLC chromatogram of the main compounds of a fresh s-TP fraction of the OMWW is shown in Figure 1. The main compounds were found to be hydroxytyrosol, tyrosol, vanillic acid, ferulic acid, and p-coumaric acid.

3.2. Isolation, 16S rRNA-based phylogeny and sequence accession

The bacterial strain from the OMWW was found to be aerobic, Gram negative, with a rod-shaped morphology and non-motile (data not shown). The primary sequence alignment carried out by NCBI

nucleotide blast search revealed that the OMWW isolate belongs to the *Acinetobacter* genus. A NJ analysis gave the phylogenetic position of this isolate closest to *Acinetobacter pittii* (Figure 2). The nucleotide sequence was submitted to GenBank, NCBI, and was assigned the accession number KY828225. For the purpose of current study, this isolate was named *Acinetobacter* REY.

3.3. Phenol biodegradation

The amount of phenol degraded and the kinetics of the process were studied at initial concentrations of 30, 50, 100 and 200 mg/L which showed a complete depletion of phenols from the culture medium after 5, 8, 8 and 10 h, respectively (Figure 3). The results show that the higher the phenol concentration, the

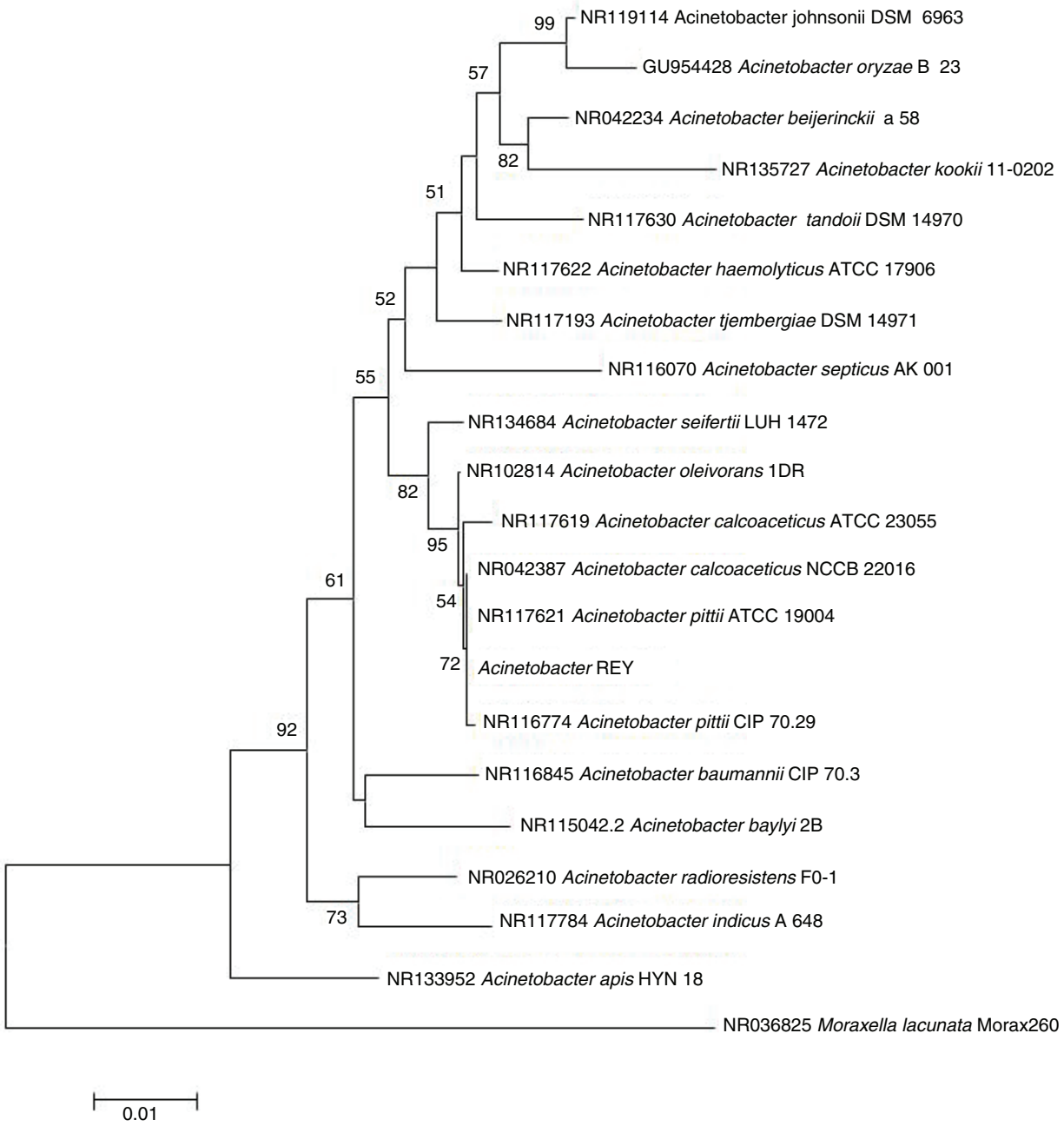

FIGURE 2. Neighbor-joining phylogenetic trees based on nearly complete 16S rRNA gene sequences of *Acinobacter* REY, an OMWW isolate, with closely related species from the genus *Acinetobacter*. Moraxella lacunata ATCC 17967 was used as an outgroup. The numbers at the nodes indicate the percentage of 1000 bootstrap replicates, values > 50% are presented. The scale bar represents 0.01 substitutions per nucleotide position.

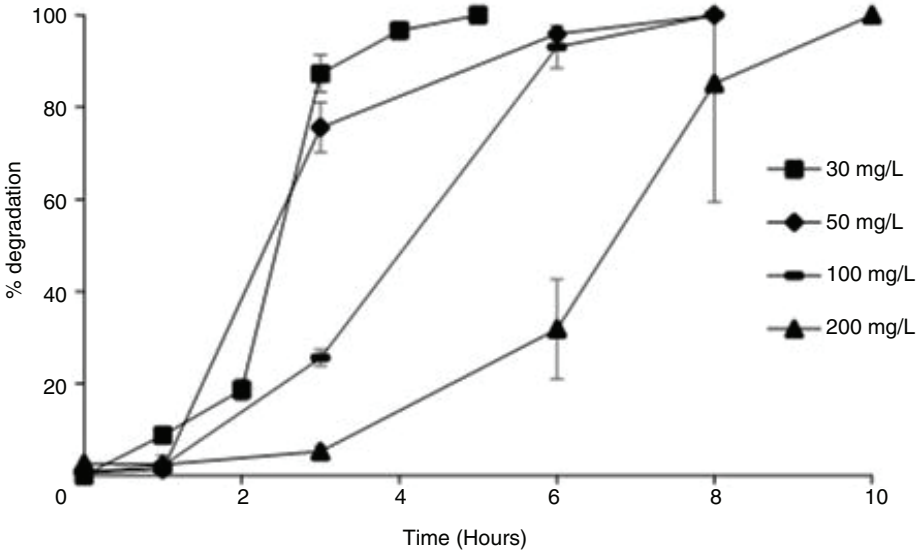

FIGURE 3. Kinetics of phenol degradation by *Acinetobacter* REY, an OMWW isolate

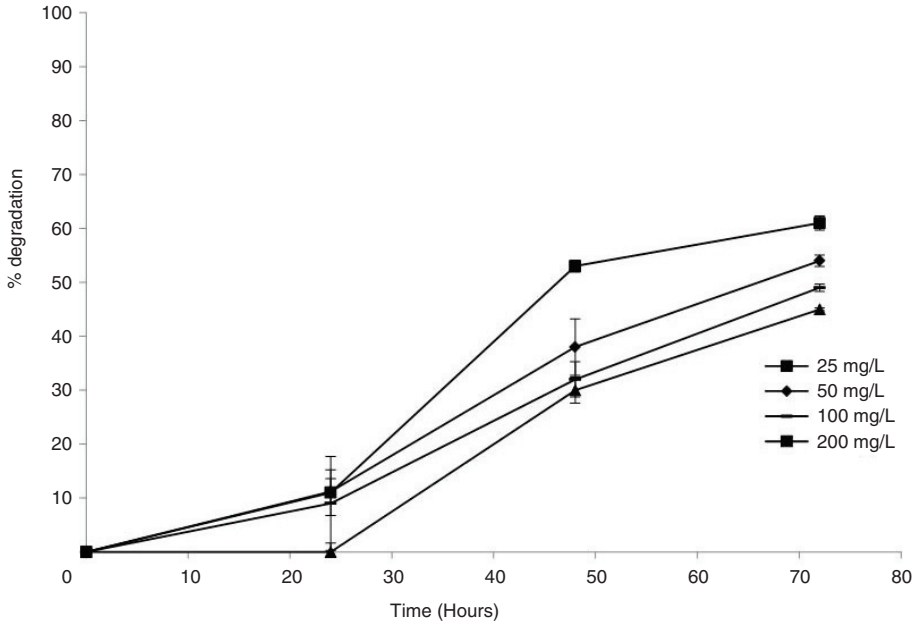

FIGURE 4. Kinetics of gallic acid degradation by *Acinetobacter* REY, an OMWW isolate

longer the time needed to fully degrade it, as found in many other studies, such as Marrot *et al*., (2006). The sterile control treatment did not exhibit any phenol loss due to abiotic adsorption or evaporation for all initial phenol concentrations (data not shown). This is in agreement with previous studies (Kurzbaum *et al*., 2010).

3.4. Gallic acid biodegradation

The bacterial strain *Acinetobacter* REY was tested for its gallic acid biodegradation kinetics. After 72 h, there was a 60, 55, 50 and 45% reduction of initial gallic acid concentrations of 25, 50, 100 and 200 mg/L, respectively (Figure 4). It was found that

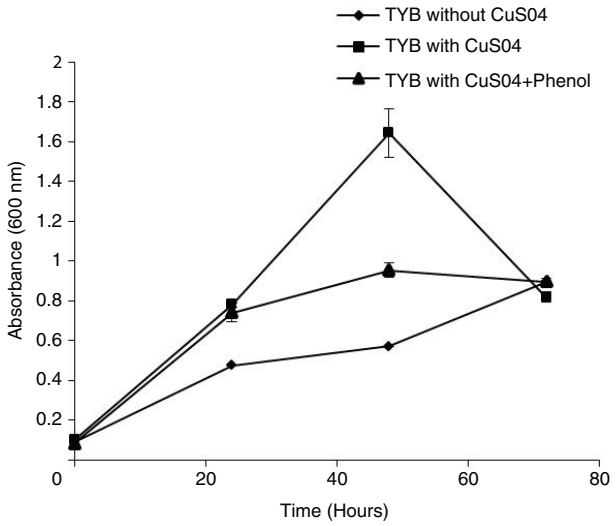

FIGURE 5. Bacterial growth in the presence and absence of the laccase inducers CuSO4 and phenols

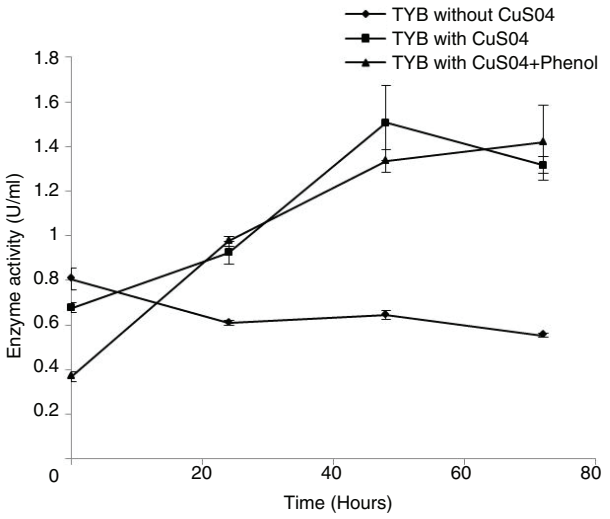

FIGURE 6. Laccase enzyme activity in the presence and absence of the laccase inducers CuSO4 and phenols

increasing gallic acid concentrations decreased the degradation rate, indicating that *Acinetobacter* REY cells were susceptible to its antimicrobial action.

3.5. Screening of the extracellular enzymes

Amylase, lipase and protease were chosen for the screening due to their broad industrial applications. Interestingly, it was found that *A.* REY was negative for all three hydrolases based on the clear zone around the colony in starch agar plates, tributyrin nutrient agar plates and skim milk agar plates, respectively (not shown). However, *A.* REY extracellular laccase was found to be positive in tryptone yeast broth in the presence of a laccase inducer.

3.6. Bacterial growth and extracellular laccase activity by OMWW isolate

The extracellular laccase extraction assay was performed with known laccase inducers ($CuSO_4$ and phenol). Figure 5 shows a typical trend where the biomass increases with time. However, it also shows that biomass growth was relatively lower in the absence of laccase inducers compared with higher bacterial growth in the presence of the inducers CuSO4 and phenols and even higher in the presence of CuSO4 alone.

Figure 6 summarizes the laccase activity in the medium, which reached a maximum of 1.5 and 1.3 U/mL with the supplemented inducers $CuSO_4$ and $CuSO_4$+phenols, respectively, after 48 h. At 72 h, minor changes were noticed for both inducers. Without any inducer, a laccase activity of 0.65 U/mL was found, which did not show any significant change over the 72 h of the experiment.

4. DISCUSSION

OMWW is one of the most polluting effluents produced by agro-food industries. It exhibits high toxicity to plants, bacteria, and aquatic organisms because of its organic substances (14-15%) and phenolic compound (up to 10 g/L) contents (Tafesh *et al.*, 2011). The organic compounds are measured as COD and phenolic content showed resistant to biodegradation. In addition, some are responsible for the OMWW's black color, depending on their state of degradation and the olive they come from (Tafesh *et al.*, 2011). The OMWW was found to contain hydroxytyrosol, tyrosol, vanillic acid, ferulic acid, and p-coumaric acid as phenolics and a high load of COD, TSS and TKN both in the crude OMWW as well as in its s-TP fraction. The presence of *Acinetobacter* species in phenolic-polluted sites is very common and well documented (Table 2). Different sub-species of this bacterium are known for their ability to degrade different pollutants (Yuan *et al.*, 2014). The 16S rRNA sequence analysis revealed that the OMWW isolate belongs to the *Acinetobacter* genus (and was named *Acinetobacter* REY) and is phylogenetically closest to *Acinetobacter pittii*.

Several authors reported on the phenol biodegradation efficacy of *Acinetobacter* species/strains. For example, *Acinetobacter* AQ5NOL 1 (Ahmad *et al.*, 2012) has the ability to eliminate 1100 mg/L of phenols; *Acinetobacter calcoaceticus* PA (Liu *et al.*, 2016) and *Acinetobacter calcoaceticus var. anitratus* (Cordova-Rosa *et al.*, 2009) showed near complete removal of 800 and 400 mg/L of phenols in minimal media, respectively. In the current study, *A.* REY completely degraded phenols (200 mg/L) within

Table 2. Different *Acinetobacter* sp. /strains and their phenol degradation efficacy.

***Acinetobacter* sp./strain**	**Phenol degradation efficacy**	**Source**	**Reference**
A. calcoaceticus var. anitratus	100% degradation of 400 mg/L of phenols after 60 h	Industrial wastewater	Cordova-Rosa *et al.* 2009
AQ5NOL 1	100% degradation of 1100 mg/L phenols at 240 h	Local site	Ahmad *et al.* 2012
A. Calcoaceticus PA	91.6% degradation of 800 mg/L phenols within 48 h	Phenolic Wastewater	Liu *et al.* 2016
A. REY	100% degradation of 200 mg/L phenols in 10 h	OMWW	Our study

10 h and at this concentration, a lag phase of up to 3 h was observed. The main reason for this is probably the phenols' toxicity. Similar results were observed in a study by Bakhshi *et al*., (2011) and suggest that high phenol concentrations might have an inhibitory effect on microbial growth.

It is shown that *A.* REY degraded gallic acid slowly with incubation times below 24 h (less than 10% from the initial concentration). Nevertheless, 48 h or more after inoculation, all four gallic acid concentrations (25, 50, 100 and 200 mg/L) underwent substantial degradation, providing compelling evidence that gallic acid can be degraded by the biomass given sufficient time for biodegradation, thus overcoming the inhibitory effect to a certain extent. This slow biodegradation kinetics of gallic acid is evidence for the relatively slow treatment process obtained in OMWW treatment plants no need to write compared to domestic waste water treatment plants. Furthermore, Figure 4 shows a 45% degradation of the initial 200 mg/L gallic acid at 72 h by *A.* REY, and that the gallic acid concentration is inversely correlated with the degradation rate. This indicates that *A*. REY cells were susceptible to antimicrobial action. This finding strongly supports the work of Borges *et al*., (2013) who showed that phytochemical constituents (for example gallic acid, tannic acid, ferulic acid and others) are antimicrobials, based on susceptibility tests that produced inhibitory concentrations in the range of 100-1000 mg/L. Borges *et al*., (2013) reported a minimal inhibitory concentration of gallic acid of 500 mg/L for *Pseudomonas aeruginosa*. However, our studies showed that *A.* REY tolerates gallic acid up to a concentration of 200 mg/L only.

The OMWW isolate was also screened for extracellular enzyme production and was found to be lipase, protease and amylase negative. However, it was shown to produce laccase, which can transform phenolic compounds and accelerate degradation processes. Our study confirmed that this isolate was able to secrete extracellular laccase, showing such a feature in an isolate obtained from an OMWW for the first time. However, the intracellular laccase production of 0.019 U/mg of protein/min after 48 h was reported in *A. calcoaceticus* (Ghodake *et al*., 2011). The laccase activity of the new isolate, *A*. REY, showed extracellular laccase activity (1.5 U/mL) that was almost identical to a pure bacterial strain of *Streptomyces cyaneus* that exhibited 2 U/mL (Margot *et al*., 2013). *Staphylococcus saprophyticus* (Mongkolthanaruk *et al*., 2012) and *Providencia rettgeri* HSL1 (Lade *et al*., 2015) were also reported as laccase producers, with lower laccase activity than *A.* REY. Many bacterial strains, for example *Bacillus tequilensis* SN4 (Sondhi *et al*., 2015), *Pseudomonas putida* MTCC 7525 (Kuddus *et al*., 2013) and *Streptomyces sviceus* (Suljić *et al*., 2015), have been reported to have higher laccase activity. Studies showed that bacterial laccase was used in the bioremediation of dye and micropollutants (Kuddus *et al*., 2013; Margot *et al*., 2013). However, little is known about the enzymatic phenol degradation which will be the future scope of our study, in order to develop an efficient bio-resource for the de-phenolization of organic matter containing high loads of polyphenols such as those found in OMWW.

5. CONCLUSIONS

In the present study, the isolation and identification of a novel laccase producing bacterial strain is presented, as the first step for the utilization of this enzyme in bio-remedial applications. Not only *A.* REY showed biodegrability of phenols and gallic acid (200 mg/L). A constitutive production of extracellular laccase, with an activity of 1.5 and 1.3 U/mL, was also found with the supplemented inducers of $CuSO_4$ and $CuSO_4$+phenosl, respectively This bacterium is thus a promising candidate for the efficient removal of phenolic compounds in phenol-rich wastewater, especially in OMWW, for which there is no satisfactory or cost-effective treatment. In future research, we will attempt to evaluate the efficacy of extracellular laccase as a pretreatment in OMWW bio-remediation, in order to protect the digestive biomass from high concentrations of phenolic compounds. It is assumed that a reduction in the phenolic concentration will enable the development of much better aerobic conditions in the OMWW medium, thus substantially increasing the biodegradation rate. This is expected to improve the quality of the effluent. The use of extracellular

laccase presents a potential horizon for the successful aerobic treatment of waste water effluents.

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