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# Isolation and Characterization of *Yarrowia lipolytica* YQ22 from Diesel Samples for Phenol Biodegradation

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Phenolic compounds have gained international interest due to their carcinogenic, toxic, and bioaccumulative properties, causing adverse effects in both animals and humans. As a result, there is a growing interest in finding alternative and eco-friendly treatment routes for phenol by exploring new microbial cultures with potential adaptation and biodegradation capabilities. In this study, the phenol removal efficiency of Yarrowia lipolytica YQ22 under laboratory conditions was determined. The YQ22 strain was obtained from diesel samples from a fuel station in Trujillo, Peru, isolated through serial dilutions on Sabouraud agar, and identified through its morphological characteristics using microscopy and molecular analysis by polymerase chain reaction of the ribosomal DNA

internal transcribed spacer (ITS) and 5.8S regions. In the treatment, the effect of pH (5, 6 and 7) and temperature (25°C, 30°C and 35°C) on phenol removal with 2% (v/v) inoculum of Yarrowia lipolytica from 48-hour growth was evaluated. Phenol concentration was measured by high-performance liquid chromatography. A maximum phenol removal percentage of 61.18 was obtained for YQ22 at 30°C, pH 5 and 120 rpm during 48 hours. These findings demonstrate the ability of Yarrowia lipolytica to remove phenol and suggest its potential use in the field of bioremediation of phenolic compounds and their derivatives.

**Keywords:** Phenol, Yarrowia, biotechnology, bioremediation, biodegradation.

## Introduction

The oil industry, along with pharmaceutical production, plastics, varnishes, textiles, and metallurgical processes, produces polluting phenolic waste (Duan et al., 2018). The presence of phenol in the environment poses a significant threat to aquatic systems and water quality (Wei et al., 2016). Phenolic contaminants and their derivatives have garnered global concern because of their carcinogenicity, toxicity, and persistence in the environment, which can have detrimental effects on both human and animal health (Zhong et al., 2018). The World Health Organization (WHO) has set a guideline that the maximum concentration of phenol in drinking water should not exceed 1 µg/L. Moreover, recent studies emphasize the critical need to treat wastewater contaminated with phenol effectively (Khan et al., 2020).

Various methods are employed to treat phenolic pollutants in industrial effluents, including chemical oxidation using hydroquinones, absorption by activated carbon, solvent extraction, and pervaporation (Nawawi et al., 2016). However, many of these processes often produce a considerable amount of intermediate and by-products. Alarmingly, some of these by-products can be even more toxic than the original contaminants, introducing secondary pollution issues that pose risks to both the environment and health. Moreover, the economic cost of certain treatments can be prohibitive and often plays a crucial role in determining the choice of treatment method (Mishra and Kumar, 2017; Nawawi et al., 2016).

Biological treatments for phenol are emerging as the most eco-friendly, sustainable methods, often outperforming conventional approaches. These methods utilize either pure microbial strains or a mix that can break down phenol and its derivatives into harmless compounds. However, pinpointing strains that can do this efficiently is a challenge (Youssef et al., 2019). Research has spotlighted bacteria like *Pseudomonas sp., Stenotrophomonas spp.,* and *Shinella spp.* – all isolated from alpine soils. They have demonstrated the ability to degrade phenol fully within 7 days at 20°C, starting from an initial concentration of 200 mg/L (Sepehr, 2019); with yeasts such as *Rhodosporidium kratochvilovae* from permafrost soils, which has the capacity to remove up to 1000 mg/L (Patel et al., 2017); and even *Acinetobacter tandoii,* isolated from the gut of lignin-decomposing termites, which can completely remove phenol from concentrations of 280 mg/L (Van Dexter and Boopathy, 2019). When subjected to real-world conditions differing from lab settings, their effectiveness can wane. A promising strategy might lie in researching native microorganisms that can offer robust bioremediation results (Gu et al., 2016).

The study in this manuscript aimed to isolate microorganisms from environments with high diesel content that would be capable of removing phenol, evaluating their performance at the laboratory level. Indeed, diesel is a fuel sold worldwide and is rich in monoaromatic hydrocarbons up to 8% (w/t) and polycyclic hydrocarbons (Frantsina et al., 2020). It contains microorganisms that may be promising in the treatment of various carbon sources and efficient phenol bioremediation (Muccee and Ejaz, 2019). There are few studies on the isolation of *Yarrowia lipolytica* from diesel with the ability to degrade phenolic pollutants; however, the literature has reported that this strictly aerobic yeast exhibits the ability to efficiently degrade hydrophobic substrates (Ferreira et al., 2023). Therefore, in this research work, *Yarrowia lipolytica* was identified, isolated from diesel sources at a gas station, and its ability to remove phenol from aqueous solutions at different temperature and pH intervals was evaluated.

# Methods

## Isolation of Yarrowia lipolytica

A 500 mL sample of diesel was collected from a gas station located in Trujillo, Peru (8.1060° S, 79.0330° W), and 10 mL of diesel was added in 200 mL of Sabouraud culture medium (SM) composed of 4.0% glucose, 1.0% peptone, 0.5% yeast extract, and 0.5% NaCl. The sample was incubated at 35°C for 48 hours, and then, using a bacteriological loop, it was streaked onto Petri plates with Sabouraud agar. The plates were incubated at 35°C for 48 hours, and colonies with distinct yeast-like morphology were isolated in pure culture. Subcultures of the purified colonies were stored at 4°C until further analysis (Benmessaoud et al., 2022).

## Phenotypic and molecular characterization

Morphological characteristics were determined using wet mount microscopy and fluorescence staining with calcein AM (Sigma Aldrich, USA) using a fluorescence microscope (ZEISS, AXIOPHOT) from a 24-hour pure culture growth (Maza et al., 2020). The isolated yeast culture was identified by molecular analysis using polymerase chain reaction (PCR) of the ITS1 region, 5.8S rRNA subunit and ITS2, following the methodology described by Saavedra et al. (2022). DNA was extracted from a 48-hour pure culture growth (Nucleic Acid Extraction and Purification Kit, innuPREP DNA Kit, AnalytikJena). The amplicon was obtained using universal primers ITS-1 and ITS-2, and the amplified rDNA was characterized on a 1.5% agarose gel. The purified PCR product was sequenced (Macrogen, USA) and aligned using BLAST (National Center for Biotechnology Information) with sequences obtained from GeneBank. A phylogenetic tree was constructed with rDNA sequences using MEGA-X software. Nucleotide sequences were aligned using ClustalW, and the phylogenetic tree was generated using the Neighbor-Joining method with the Tamura 3-parameter model and a Bootstrap consensus of 1000 replicates. A total of 226 positions were shown in the final dataset (Iurescia et al., 2022).

## Preparation of phenol solution

A chemically pure phenol solution (Merck, USA) was prepared at a concentration of 10 000 ppm. Five grams (5 g) of phenol were weighed and dissolved in 500 mL of 0.02 N NaOH, gently stirred until completely dissolved (Cruz et al., 2021).

## Preparation and adaptation of the inoculum

The inoculum was prepared from a 48-hour Sabouraud broth yeast culture of 200 mL. Cells were recovered by centrifugation at 6000 rpm for 5 min and resuspended in a 0.85% NaCl solution. Optical density was adjusted to 0.2 at 600 nm using a UV/VIS spectrophotometer (Evolution™ 260 Bio, USA) (Almajali et al., 2021).

A 50 mL Sabouraud broth supplemented with 50 ppm of phenol was prepared and inoculated with 2% (v/v) of the prepared culture. The mixture was incubated at 35°C for 48 hours. Once growth was obtained in 50 ppm of phenol, the culture was inoculated into a broth with 100 ppm of phenol, and so continually, until a concentration of 1000 ppm of phenol was reached (Filipowicz et al., 2020).

## Phenol biodegradation assay

The experiments were conducted using a multi-level factorial design with two factors: pH (5, 6 and 7) and temperature (25°C, 30°C and 35°C), for a total of 27 experiments in 150 mL flasks with a working volume of 50 mL of MS base medium (2.0% glucose, 1.0% peptone, 0.5% yeast extract, and 0.5% NaCl) supplemented with phenol at an initial concentration of 1600 ppm. The inoculum was 2% (v/v) of the prepared *Yarrowia lipolytica* culture from a 48-hour young growth and adapted previously. The mixture was incubated at 120 rpm for 48 hours, and biomass was monitored every 12 hours. Finally, the residual phenol concentration was determined after incubation (Al-Tarawneh et al., 2022).

## Analytical methods

The cell concentration was determined by optical density (OD) at 600 nm using a UV/VIS spectrophotometer (Abdulrasheed et al., 2020). The residual phenol was determined by high-performance liquid chromatography (HPLC) using a UHPLC system, Thermo Scientific (Dionex Ultimate 3000, USA) with a UV/Vis DAD detector (Wen et al., 2020).

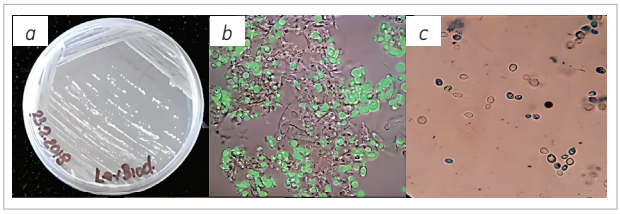
## Statistical analysis

The obtained data were subjected to an analysis of variance (ANOVA). The treatment means were analyzed using a Tukey test at a significance level of 5% using the computer software Minitab 18 (Khalil et al., 2021).

## Results and Discussion

A typical yeast colony (assigned as YQ22) with a smooth and shiny appearance was isolated from the gasoline station diesel. Microscopic observation under fluorescence at 1000X magnification showed pseudohyphae formation and revealed the presence of oval-shaped yeasts (*Fig. 1*). The colonies of YQ22 exhibited a round shape, regular edges, a creamy appearance and a rough surface.

**Fig. 1.** *Morphological observation of the isolated yeast pure culture: (a) colonies of yeast culture on Petri dishes with Sabouraud agar, (b) fluorescence microscopy of the yeast culture with a total magnification of 1000X, and (c) observation in fresh condition with 0.1% lactophenol blue*




The YQ22 strain was identified through PCR using the regions of the 5.8S rRNA, ITS 1, and 2 genes, obtaining an amplicon with a size of 303 bp, which was revealed by agarose gel electrophoresis 1.5% (*Fig. 2*). Likewise, the obtained sequence was compared to the sequences of the GeneBank database and showed a similarity of 93.17% with Yarrowia lipolytica CBS 6124 (T), which was also identical to five other Yarrowia lipolytica sequences included in the phylogenetic tree (*Fig. 3*). The annotated sequence was deposited in the NCBI database with accession number OQ362266 (https://www.ncbi.nlm.nih.gov/nuccore/OQ362266). These results are consistent with what was reported by Al-Dhabaan (2022), who found strains that degrade aromatic hydrocarbons such as Rhodotorula ingeniosa, Rhodotorula mucilaginosa, and Candida parapsilosis from isolates. Previously, strains of Yarrowia lipolytica with degrading activity of aliphatic hydrocarbons have also been reported, isolated from areas contaminated by petroleum (Hassanshahian et al., 2012).

**Fig. 2.** *Agarose gel electrophoresis (1.5%) of the ITS region amplification from sample N03. CE: Extraction Control, C-: Negative Control, C+: Positive Control, M: 1Kb marker*

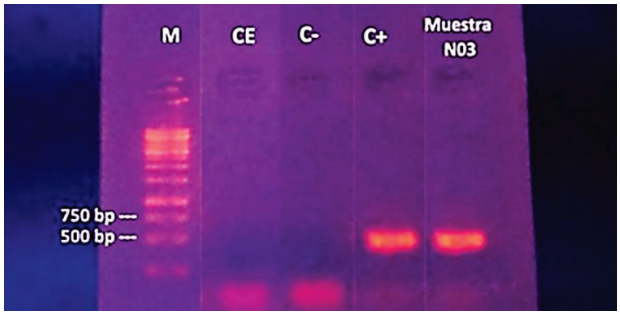
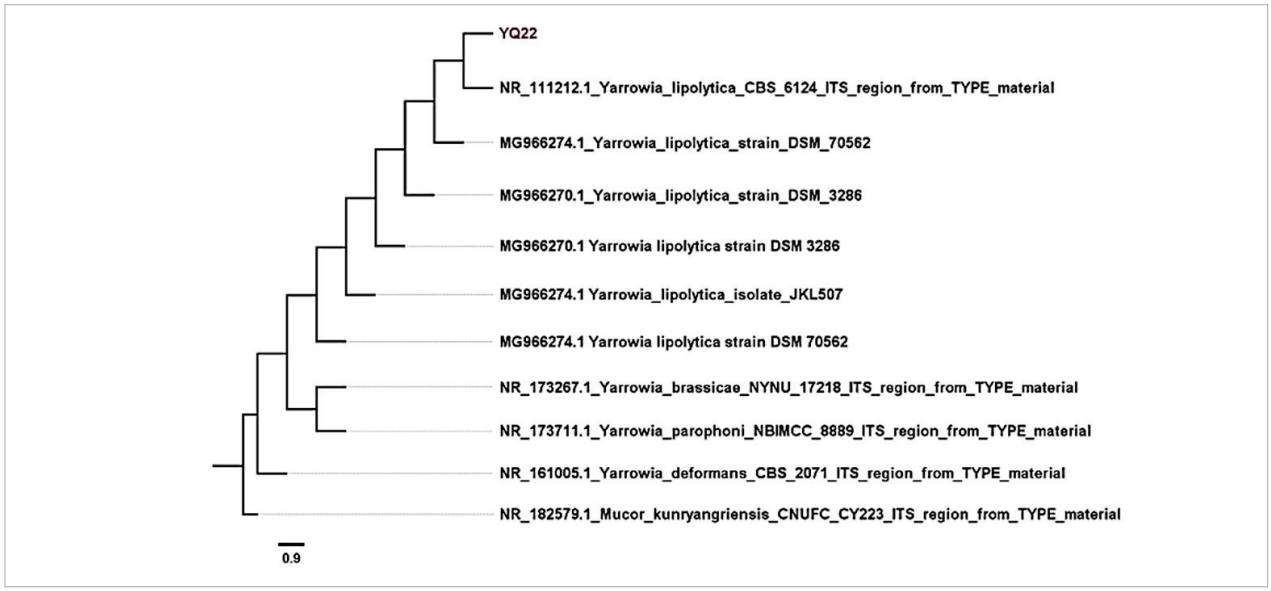



**Fig. 3.** *Phylogenetic analysis established from the ITS sequence obtained from YQ22 strain isolated from diesel fuel of a gas station. Mucor kunryangriensis was used as an outgroup. The bar shows a sequence divergence of 0.9%*

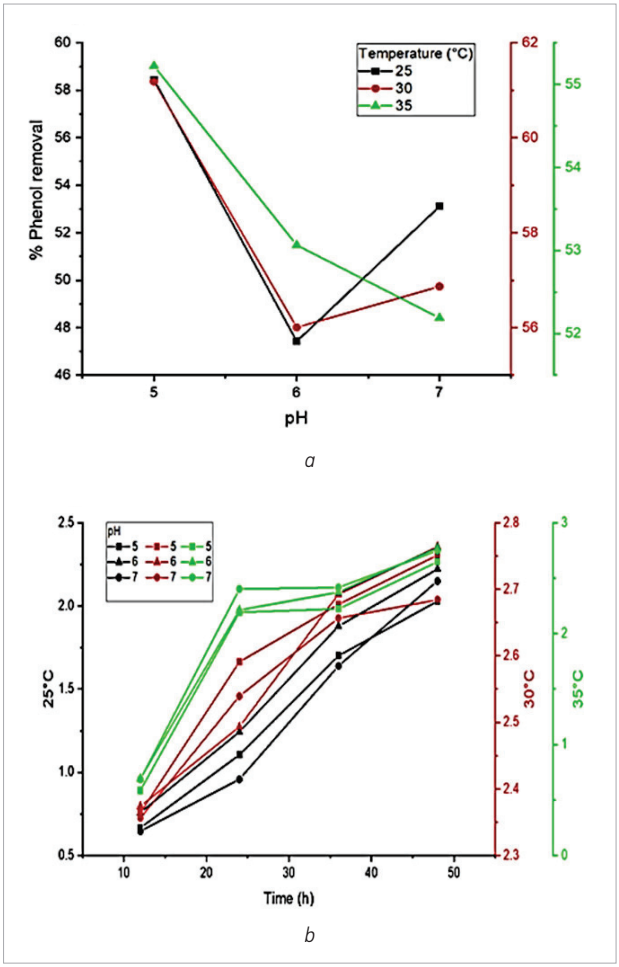


The maximum percentage of phenol removal by *Yarrowia lipolytica* YQ22 was 61.18% at 30°C and pH 5 with 120 rpm for 48 hours (*Fig. 4*). These results differ from Mahgoub et al. (2023) who achieved degradation of up to 74.67% of phenol from 1000 mg/L for three days using *Pseudomonas aeruginosa, K. pneumoniae* and *K. variicola.* However, it is slightly similar to Wen et al.'s (2020) findingof a removal of 67.10% phenol at pH 7 for 42 hours using *Rhodococcus sp.* Moreover, pH showed a significant difference on the percentage of phenol removal among the experimental assays ($P < 0.05$) with a value of 0.01. This is because pH affects the physiological and biochemical activities of the microorganism, influencing its growth and proliferation (Aisami et al., 2020). In *Fig. 4*, it can be observed that at an initial pH of 5, a higher percentage of biodegradation was observed at different temperatures. Under the same conditions, it has been found that successful phenol biodegradation ranges between pH 5 and 6, and below or above that range, the biodegradation of phenols tends to decrease (Bernats and Juhna, 2015); likewise, a maximum cellular biomass (OD600) of 2.7 was obtained at 30°C, although no significant difference was observed with the other treatments (*Fig. 4*). It has been reported that *Yarrowia lipolytica* shows an optimum pH for the degradation of aromatic compounds within the range of 5–6 (Dunoyer et al., 2014).

Furthermore, temperature showed a significant difference of 0.028 among the treatments ($P < 0.05$), indicating that maximum phenol degradation occurred at 30°C. This is due to the fact that temperature also influences enzyme activities, playing a role as important as, or even greater than, available nutrients during the degradation of organic compounds, including phenol (Aljbour et al., 2021). *Fig. 4* shows higher biodegradation of phenol at a temperature of 30°C, which is similar to what was demonstrated by Lee et al. (2012) who reported a temperature range between 30°C and 40°C as optimal for phenol reduction by a laccase from *Yarrowia lipolytica.* Similarly, Hackenschmidt et al. (2019) mentioned in their study that the recommended growth temperature for *Yarrowia lipolytica* was 25°C to 30°C.

Therefore, *Y. lipolytica* YQ22 isolated from a diesel sample obtained from a fuel dispensing establishment showed biodegradative activity for phenol, contributing to future technologies in the bioremediation of phenolic pollutants from contaminated natural sources (Tarón et al., 2014).

**Fig. 4.** *Graph of factor interaction (pH and temperature) on (A) the % removal of phenol and (B) cell growth by Yarrowia lipolytica YQ22*




## Conclusions

A YQ22 yeast culture was obtained from diesel samples, capable of degrading phenol in the aqueous medium up to 61.18% at 30°C, pH 5, with 120 rpm for 48 hours. The yeast was identified as *Yarrowia lipolytica* based on its morphological and molecular characteristics. It demonstrated its potential for phenol degradation by tolerating up to 1600 ppm of the compound, as well as exhibiting a short biodegradation time and higher cellular biomass growth. Therefore, *Yarrowia lipolytica* YQ22 serves as an alternative biological model in the biodegradation of phenolic pollutants and can be applied in the bioremediation of effluents contaminated with phenol.

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