BIODEGRADATION OF PHENOL AT HIGH CONCENTRATION BY A NEWLY ISOLATED ADAPTED AND IDENTIFIED BACTERIA *LECLERCIA ADECARBOXYLATA*

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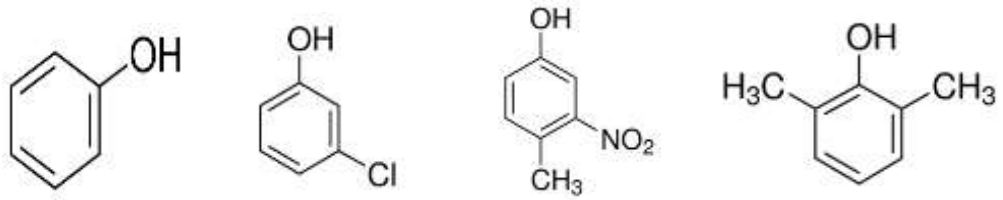
Abstract: Wastewater treatment has become very important due to insufficient water resources. For wastewater treatment, Physical, Biological, and Chemical methods have been developed. Bioremediation is the most efficient and environmentally sustainable process among them. This article presents the continuous decomposition of phenol-enriched refinery effluent by stimulated, activated, and adapted granules of bacteria and yeast cultures. A novel *Leclercia adecarboxylata* has been revealed to be capable of degrading phenol at high concentrations as well as its potential for bioremediation of phenolic wastewaters. Water samples were collected from the first and second bio-based basin of the refinery in the Republic of North Macedonia to observe microbial growth. Microorganisms were extracted from refinery and synthetic wastewater. The whole procedure was performed in laboratory bioreactors under defined parameters. Several colonies of bacteria and yeast capable of degrading petroleum wastewater and phenol were isolated and adapted from active sludge originating from the treatment system of oil refinery. Phenol concentration was gradually increased from 100 to 2000 mg/L^{-1} under laboratory conditions. All isolates were incubated with refinery wastewater for 20 days at 35 ^{o}C and phenol degradation was monitored. 4-aminoantipyrine in the colourimetric assay method according to standard methods reported by the United States Environmental Protection Agency (EPA) was used for measuring the changes in phenol concentration. Final Strains were grown in batch cultures in 250-ml flasks containing 50 ml of MSM supplemented with phenol (1500 mg/liter utilized phenol as the sole carbon source and energy. Totally 9 isolates of 5 bacteria (BF), and 4 yeasts (KvF) were identified as; *Leclercia adecarboxylata*, *Bacillus subtilis*, Citrobacter sp., Raoultella sp. In conclusion, it was identified that BG BF-1 was the most resistant and durable bacterial strain which can degrade and adapt at high phenol concentration. Phenotypic profiling and sequence analysis identified the strain as *Leclercia adecarboxylata*.
The resistance and viability of Leclercia adecarboxylata at high level of phenol concentrations indicates that this would be used for phenol biodegradation.
Cultivation of phenol degrading Granulated forms of Leclercia adecarboxylata with other bacteria and yeast cultures can be an alternative to phenol bioremediation treatment strategy.
Keywords: Leclercia adecarboxylata, Phenol- Biodegradation, Wastewater.

1. INTRODUCTION

The primary sources of pollution of our rivers lakes and seas are densely populated cities (with the unimpeded arrival of the population from inland to overcrowded places anyway) (Hanafi and Sapawe, 2020).Human life is threatened due to the rise mostly in number of chemical industries, oil refineries, coke ovens, and livestock farms, and the leakage of untreated sewage into urban sewers.
Thus, various inorganic and organic compounds are constantly approaching our natural waters, the majority of which are not degradable and are toxic to humans and animals. Toxic compounds include phenol, which in some plants serves as raw material, while in others it serves as a final product or by-product. (Silva et al., 2012)
The phenol commonly used in such industries is an extremely volatile, carcinogenic, mutagenic, and teratogenic agent with major environmental impacts. The United States Agency for Environmental Protection has classified it as a priority pollutant (Evans & Frank, 1979). Priority chemicals containing phenols, especially chlorophenols and nitrophenols, have been identified by the US Environmental Protection Agency (EPA) and the European Union. The compositions of major phenols considered the most important pollutants by the EPA are shown in Figure 1.

Figure1. Chemical structures of phenols, Carbolic acid Phenol and Hydroxybenzene



Phenol chlorophenols nitrophenols Dimethylphenol

Friedlieb Ferdinand Runge, a German chemist, extracted phenol from coal tar in 1834 and called it "Karbolsäure" (carbolic acid or coal-oil, remained the primary source until the development of the petrochemical industry in 1841). Phenol and hydroxybenzene are aromatic compounds which can be either synthetically or naturally.

Phenol with the chemical formula C_6H_5OH, also known as phenic acid, is an organic compound that is stable at room temperature and is stored in a white-crystalline state. It is a colourless, opaque crystal mass that seems to be made of white powder. When combined with water, it becomes a syrupy liquid that turns pink to red in the atmosphere. Phenol is soluble in alcohol, glycerol, petroleum, and water and has a sweet tar-like odour (Mohd A., 2020). Phenolic compounds were used as creosote in their raw state to prevent corrosion of railway and ship timber and to reduce smell and decomposition. Scientists have discovered that phenol could be used to make dyes, aspirin, and, among the most volatile acids, picric acid. Aromatic compounds are broken down into a few substrates by bacteria, such as catechol, protocatechuate, and gentisate, which are several phenolic compounds that are converted. (Mahammedilyas et al., 2010; Bui et al., 2012).

Phenol can be found naturally in particular cereals, livestock manure, and organic substances decaying naturally. It is a strong air pollutant that can damage structures and ozone layers and can reduce the atmospheric visibility and heat balance. (Bui et al., 2012) The United States Environmental Protection Agency (US-EPA 2006) has therefore declared phenol to be a harmful substance and dangerous environmental pollutant.

In different quantities, phenol can be present in surface water. It was detected in amounts ranging from 0.01 to 2.0 g/L in water samples, but it is important to note that in river water polluted by sewage from petrol processing plants, the concentration can reach or exceed 40 mg/L. EU Directive 2455/2001/EC specifies an overall concentration of 0.5 g/L in drinking water, and it should be lower than 0.1 g/L in drinking water. (Michałowicz and Duda., 2004)

Phenol has been shown to have negative impacts on human health and the environment. When ingested, inhaled, or encountered by humans or other living things, phenol is a water-soluble neurotoxin that can cause harm. People have been exposed to phenol in toxic doses of 2 mg and 4 mg a day, respectively, by chronic ingestion of refined foods with high phenol content or inhalation of air from highly polluted locations. Exposure to phenol can also be unavoidable. The analysis also shows the emitting of about 0.3–0.4 mg phenol in a single burning cigarette.

Related to the use of phenol-polluted groundwater, daily exposure of about 10–240 mg of phenol per person has been reported. Exposed to the high phenol content, the diarrhea dark urine, and oral inflammation grew clinically considerable. (Mohd A., 2020; W. Michałowicz & Duda., 2007).nAs one of the most life-threatening toxicants, phenol must not be allowed to pollute nature and damage life forms; rather, it must be properly removed and disposed of. Bioremediation, a pure, easy-to-make degradation process, is one of the easiest and most cost-effective methods. On the assumption that phenol is a source of carbon and energy for many micro-organisms, the current treatment methods are inefficient compared to the natural and energy-saving processes of phenol biodegradation (Kavilasni et al., 2019).

It has been documented that native microbial species are more adaptive than emerging non-native microorganisms. Therefore, for the bioremediation of phenol-contaminated areas in different regions, it is necessary to identify new phenol-degrading bacteria. Many types of yeast may also use aromatic compounds and phenol. Many studies have demonstrated the biodegradation efficiency of pure or mixed cultures of microorganisms, demonstrating the potential for industrial effluent treatments based on their ability to grow and utilize phenol present in wastewater, particularly wastewater from petroleum refineries. Numerous findings have shown that the ability of pure or mixed cultures of microorganisms including Pseudomonas, Bacillus, Escherichia, Acinetobacter, and Corynebacterium species to biodegrade phenol in wastewater, especially in petroleum refineries, clearly shows their application in industrial effluent treatments. (Nwanyanwu & Abu, 2012; Sachan et al., 2019; Zhenghui et al, 2016)

In this study, we report the isolation of several bacteria and yeast species, with a particular focus on an enteric bacterial strain, BIG FB- that can degrade high concentrations of phenol as a sole source of carbon. Phenotypic profiling and sequence analysis identified the strain as *Leclercia adecarboxylata*. Many records of phenol

degradation by various organisms have shown in the literature, this will be the first report of phenol degradation by *L. adecarboxylata*.

This research provides a potential alternative method for phenol bioremediation via microbial isolation and adaptation. Degradation of refinery wastewaters is investigated using the isolation and characterization of naturally occurring microorganisms from wastewater and activated granulated microorganisms.

2. MATERIALS AND METHODS

For the enrichment culture, phenolic wastewater raw samples were collected from bio-based aeration basin of the refinery in the Republic of North Macedonia and inoculated into flasks containing MSM (minimal salt medium,). The MSM contained; KH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $CaCl_2$ 0.1 g, NaCl 0.2 g, $MgSO_4$_7H2O 0.5 g, $MnSO_4$_$7H_2O$ 0.01 g, $FeSO_4$_$7H_2O$ 0.01 g, NH_4NO_3 1.0 g per litre. The phenol-using microorganisms for the sample were grown in 250 mL Erlenmeyer flasks containing 100 mL of sterile nutrient broth media (HIMEDIA)100 mg/L^{-1} and phenol were added to the mineral substrate. 250 ml cultivation was placed in 2 L refinery wastewater and aerated for 24 h.

This was done in several phases of 24 hour periods and phenol concentrations were observed at the beginning and at the end by biological and chemical parameters. The laboratory bioreactor used in all processes was made up of an aerobic-a nitrifying section and an anaerobic-denitrifying section.

Figure 2. (a) MSM preparation (b) inoculation of microbial cultures

(a) (b)

Bacteria were isolated using TSA medium (Tryptic Soy Agar), while yeasts were isolated using YP (Yeast peptone) and RBC (Rose-Bengal Chloramphenicol Agar). At 30 degrees Celsius, the plates were incubated (Figure 2). Centrifugation at 6000 rpm for 10 minutes was used to obtain the cells. The harvested cells were washed twice in sterile phosphate buffered saline (PBS, 0.02M) to resuspend in the same medium and the turbidity was spectrophotometrically calibrated to provide an optical density of 0.6 at 540nm.Their determination was carried out according to standardized methods. The phenol-degrading bacteria and yeasts were purified and preserved in nutrient agar medium at 4^{o}C within a week of growing on mineral salts-phenol culture medium. For further analysis, pure strains were inoculated on slag agar with paraffin and stored at 4 ° C.

A control consisting of wastewater without inoculum was set up for cell growth and biodegradation of the phenolic effluent. The optical density (OD) and total viable count were monitored on a regular basis to measure the use of phenol and the microorganisms' growth profiles in phenolic wastewater. Supernatant analyses and the isolation of selected microorganisms were performed before and after 24h for the each aeration for a week.

Isolated microbial cultures were added with 250 ml of MSM (mineral salt media) and gradually increasing an appropriate concentration of phenol: 50, 100, 200, 300, 500, 800, 1000, 1200 mg/L^{-1}. The resulting cultivation was aerated and circulated throughout the water bath shaker for 72 h, 150 VM at a temperature of 28 °C ± 1 °C.

Phenol concentration of the sample was determined by using the 4-aminoantipyrine in the colorimetric assay according to standard methods (Spectrophotometric, Manual 4-AAP with Distillation) reported by the United States Environmental Protection Agency (EPA). After centrifuging the samples, 4ml of supernatant was removed and transferred to screw-capped tube. The tubes were supplied with one fifth (0.2 mL) of 2N NH_4OH, supplemented by 0.1ml 2.0% (w/V) 4-aminoantipyrine and 8% (w/v) potassium ferricyanide 0.1ml [K_3Fe (CN) 6]. The phenol samples were balanced at pH 10.0 and 4-aminoantipyrene was added in the presence of ferricyanide ion to obtain a yellow or amber colour complex. Extraction of the compound into chloroform increases the colour intensity. The phenol content of the sample is determined quantitatively by measuring its colour. (Chitra et al., 2018) The absorption of the tubes was measured at 500 nm and the phenol level was evaluated. The phenol-degrading strains usual physiological and biochemical features, such as Gram's staining and motility, were assessed using standard manual procedures. Optical microscopy was used to examine the morphological features of the isolated colonies.

3. RESULTS AND DISCUSSIONS

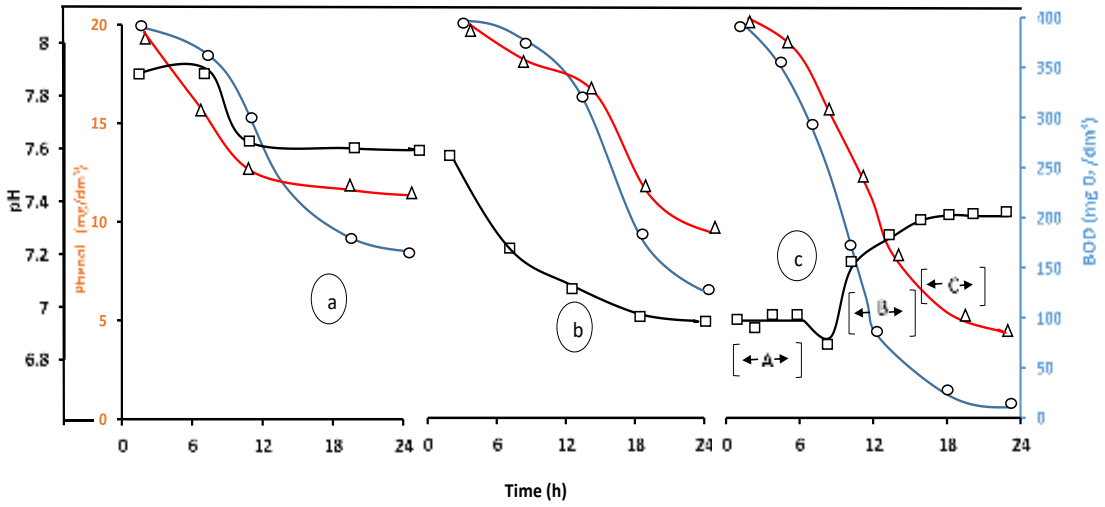
In activated sludge, as a natural habitat for microorganisms that are in direct contact with wastewater containing phenol and other components, a natural selection of microorganisms capable of degrading phenol takes place. In order to create an efficient mixed microbial culture for such degradation, wastewater and sludge samples were gathered from an oil refinery output in North Macedonia. (Figure3)

For the enrichment and isolation of phenol-degrading bacteria and yeasts, samples were inoculated in phenol-containing medium. For the proper isolation and characterisation, incubation procedures were performed repeatedly

Figure3. Isolated Bacteria and Yeast Samples

The phenol content, BOD, pH, and sludge deposition rate were all determined during the active sludge degradation testing. The activated sludge was reactivated in a laboratory bioreactor using effluent from the refinery. The capacity of microorganisms to convert phenols into biomass was measured by reducing the phenol content in the artificial nutrient medium with phenol, and pH variations represented full oxidation of phenols to products (Figure 5).

Figure 5. Phenol degradation in activated sludge from oil refinery followed by changes in pH (-□-), phenol concentration (-Δ-) and BOD (-o-). (a, b, c) refer to changes in pH.



Following three adaptive cultivations of activated sludge, biomass, and wastewater separation by sedimentation, the third cultivation showed the most noticeable modifications. After 18 hours, phenol was consumed, the BOD was reduced from 400 mg to 50 mg O_2/dm^3, and pH changes meant a change in the ratio of yeast and bacterial strains in the sludge, confirmed by microscopic examination. Approximately 60% yeast to 40% bacteria was formed in the activated sludge, which was confirmed by their cultivation in Petri on agar medium. During the phenol degradation for another 6 hours, the pH of the wastewater increased slightly compared to the first and microscopic examination showed that the sludge contained about 20% yeast and 80% bacteria. This ratio of bacteria and yeast was maintained until the end of adaptive cultivation.

For three weeks of microbial growth, isolates were collected from 300 mg/l of the phenol plates and inoculated to Muller-Hinton and YP agar plates. 9 isolates including five bacteria (BF) and 4 yeasts (KvF) were collected after 72 hours of cultivatedion (Figure 6). All these strains used phenol as the only source of carbon and energy.

Figure 6. The morphological properties of the isolated colonies by optical microscopy (A) Yeast Colonies (B) and (C) Bacteria colonies

A) (B) (C)

Table1. Isolated Bacteria and Yeast Species

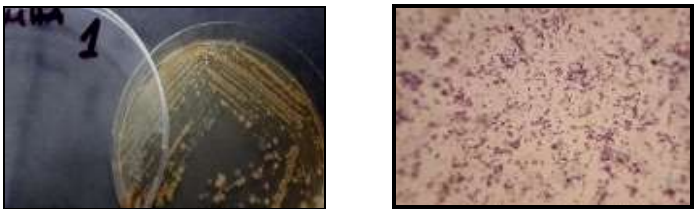
Aquisition Time	Name	Sample	%	Family	Genus	Species
12:28	AE0379_0001_I10[c]	II BF-1	99.9	Family I Enterobacteriaceae	Leclercia	adecarboxylata
10:47	AE0403 _0001_J3[c]	II BF-2	90.1	Family I Enterobacteriaceae	Raoultella	sp.
12:28	AE0379_0001_K10[c]	II BF-3	99.9	Family I Enterobacteriaceae	Citrobacter	sp.
12:28	AE0379_0001_L10[c]	II BF-4	-	-	-	-
12:28	AE0379_0001_M10[c]	II BF-5	81.9	Family I Bacillaceae	Bacillus	subtilis

Two Bacillus Gram+ bacteria, one Coccobacillus Gram+ bacteria, and one Big-rod shaped Gram+ bacteria were the morphological characteristics of the isolated bacteria and yeast colonies (Figure 7). Yeast colonies demonstrated the usual physiological and biochemical characteristics which yeasts are identified for.

Figure7. Morphological and Microscopic Wives of Isolated and Adapted Samples

Microbial identification was done by MALDI-TOF/Saramis. Single cell colony from agar plate (incubated from 16-24 h) is transfer to the MALDI steel plate, Axima 384x2.8mm target plate (DE1580TA, Kratos Analytical Limited and Shimadzu Corporation). The cells are immobilized with addition of 1 µl matrix (40 mg/ml α-Cyano-4-hydroxycinnamic acid (CHCA) in water/acetonitrile/ethanol (1:1:1) with 0.03% trifluoroacetic acid) and air-dehydrated within 10–15 min at room temperature. The reference strain *Escherichia coli* DH5α is used as a standard for calibration and as reference for quality control. The protein mass profiles (spectrum) is obtained using Linear acquisition mode of the MALDI-TOF-TOF mass spectrometer (Axima Performance, Shimadzu Corporation), with laser power of 56 V, frequency of 50 Hz and data acquisition range from 2000 to 20000 Da. Peak list obtained in form of ASCII file is transferred directly into the SARAMIS software, where the pattern is compared against the SARAMIS database and subsequently the microorganism is identified up to species level (Giebel et al., 2010).

Figure8. (A) Microscopic (B) Morphological Properties of Leclercia adecarboxylata BIG FB-1



Leclercia adecarboxylata is an aerobic, Gram-negative, rod-shaped, opportunistic human pathogen of the Enterobacteriaceae family. The name *Leclercia adecarboxylata* has been suggested for a group of bacteria in the Enterobacteriaceae family that was previously known as Escherichia adecarboxylata. All other Enterobacteriaceae species may be distinguished phenotypically from *Leclercia adecarboxylata*. This species' members exhibit motility, indole synthesis, methyl red, and growth in the presence of KCN (Tamura et al., 1986).

5. CONCLUSIONS

Because of its long-lasting and harmful effects, phenol treatment from industrial effluents is essential. From the oil refinery effluent, different colonies of yeast and bacteria capable of degrading high phenol concentrations and petroleum wastes were identified and modified. The combination of them with resistant strains of native microorganisms was provided to obtain adapted granules with greater ability for phenol biodegradation. Batch toxicity studies revealed that the strains and granules could resist the toxicity of the phenol at concentrations of up to 2000–2500 mg/L and had a significant potential applicability in the treatment of phenol-containing contaminants.

In particular, a novel bacterial strain that can degrade phenol at high concentrations was isolated from the petroleum effluent in North Macedonia. Phenotypic profiling and sequence analysis identified the strain as *Leclercia adecarboxylata*. In natural environments polluted by industrial toxic waste, including hydrocarbon or petrochemical residues, different kinds of microorganisms can be detected. *L. adecarboxylata* granules could collaborate with other native phenol-resistant microorganisms in granular form, which would result in greater ability to biodegrade phenol. In conclusion, *Leclercia adecarboxylata*, a bacterial strain that can break down phenol at both low and high concentrations, was isolated for the first time.

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