ISOLATION ADAPTATION AND DETERMINATION OF *BACILLUS SUBTILIS* FOR BIODEGRADATION OF PHENOL FROM WASTEWATER

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Abstract: Despite the insufficient water resources, the growth of the population and the rapid development of the industry have made wastewater treatment important. Several methods have been designed for phenol removal. Among them Bioremediation is the most effective, environmentally friendly and low-cost method. This study presents an alternative method to achieve bioremediation of phenol using microbial isolation and adaptation. It was aimed to monitor the viability and resistance of *Bacillus subtilis* to the high phenol concentration and the possible use in the bioremediation. Monitoring the phenol as the main target, other parameters were also followed as well as the interaction of *Bacillus subtilis* with other granulated bacteria and yeast. It was aimed if different bacteria species and yeasts can collaborate with *Bacillus subtilis* to degrade phenol. Water samples were collected from the last refinery treatment cell to observe bacterial growth. Water samples were tested for several parameters such as dissolved O_2, Temperature, pH, Salinity, NH_4 and P. Phenol degrading microorganisms were isolated from wastewater and synthetic water. Analyzes were performed by supplementing the phenol concentrations from 100 to 2000 mg/ L-1 In MSM and 72 h aeration-agitation at 150 rpm/ 28^{o}C. 4-aminoantipyrine in the colourimetric assay method was used for phenol concentration according to standard methods reported by the United States Environmental Protection Agency (EPA). The morphological properties of the isolated colonies by optical microscopy were; Bacillus Gram+ bacteria, Big-rod shaped Gram+ bacteria and typical yeast colonies. Microorganisms were identified as; *Bacillus subtilis, Leclercia adecarboxylata, Citrobacter sp., Raoultella sp.* All isolates were incubated with refinery wastewater for 20 days at 35 ^{o}C and phenol degradation was monitored. The resistance of microorganisms dramatically decreased as phenol concentration increases. In conclusion, *Bacillus subtilis* strain was isolated and adapted to a high concentration of phenol and their determination for Biodegradation of Phenol. The tolerance and viability of *Bacillus subtilis* at high concentrations of phenol, promises the potential for its utilization for bioremediation of phenol in the wastewater and petrochemical industry. Granulated-combined of *Bacillus subtilis* with other microorganisms can be an alternative for phenol bioremediation treatment strategy. Cultivation of phenol degrading microorganisms in oil-contaminated environments will help the restoration of ecosystem balances.
Keywords: *Bacillus subtilis*, Phenol-degradation, Biodegradation, Wastewater.

1. INTRODUCTION

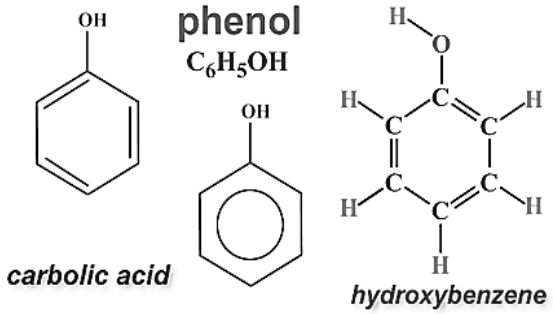
Industrial development and dramatic increase of the world population resulted more water consumption. The increase in water demand and the reduction in water resources have made the waste water treatments more important.

Waste water can be reused if it passes through wastewater treatment units, sometimes additional treatment is required for the removal of suspended solids and other contaminants. Water that has not come in direct contact with hydrocarbons, or one that has been minimally contaminated by such chemicals, can be a source of reuse in industrial processes. Refineries typically generate significant amounts of wastewater that are in contact with hydrocarbons (McGraw-Hill, 2008; Dursun, & Tepe, 2005).

There are several strategies for managing wastewater in the petrochemical industry which includes procedures that contribute to reducing the amount of used water for the needs of the industry. Among the all process, biological

treatment is one of the most effective, environmentally friendly and energy-saving method for removing pollutants of industrial wastewater (Markov & Georgievska, 2016; Gacesa & Klasna, 1994). Many compounds including phenolic compounds at high concentrations are commonly found in the wastewaters discharged from several important industrial processes such as coke production, oil industries, petrochemicals manufacturing, textile and coal industries, petroleum refinement, pharmaceuticals, plastic industries and landfill leachate. These wastewaters have a serious environmental impact due to their high complexity and toxicity (Feng et al., 2015; Silva et al., 2012).
Among others, the phenol widely used in these industries is a highly toxic, carcinogenic, mutagenic and teratogenic substance that has an extraordinary environmental implication. (Laowansiri et al., 2008; Celik et al., 2008). It is listed in the priority pollutants by the United States Agency for Environmental Protection (Evans & Frank,1979) Hydroxybenzene and carbolic acids are highly toxic intermediates in chemical synthesis used as a germicidal agent which causes serious corrosive to the skin. There is a necessity to remove phenolic compounds from the environment.
Phenol including aromatic compounds are spread in nature and released phenols are metabolic by products of decomposition of plant residuals. Decaying of organic matters like rotting vegetables and coal are other natural phenol producers in environment. Naturally produced phenol can be produced by fractional distillation of coal tar, 1-ethylethylbenzene (cumene) is the most source of phenol which can be used as an indication of the levels of phenol production. Phenol was isolated from coal tar in 1834 by the German chemist, Runge and named karbolsaure (coal-oil acid or carbolic acid though its composition was not known until 1841). Phenol and hydroxybenzene (Figure1) are both a synthetically and naturally produced aromatic compound.

Figure 1. Chemical structures of phenols, Carbolic acid Phenol and Hydroxybenzene



Phenolic products were first used in raw state as creosote to prevent the weathering of railway and ships timber to reduce the odour and decomposition. Scientists have found applications of phenol in the synthesis of dyes, aspirin and one of the high explosives picric acid. In bacteria aromatic compounds are converted to few substrates. Phenolic compounds that are converted via bacteria are catechol, protocatechuate and rarely gentisate.
Phenol is a colourless, translucent, crystalline mass, white powder at room temperature. It turns syrupy liquid when mixed with water and turn pink to red in air. Phenol has a sweet tar like smell and is soluble in alcohol, glycerol, petroleum and water (Mahammedilyas et al., 2010). Based on the theory that some microorganisms can use phenol as a source of carbon and energy, a new energy-saving and inexpensive process of biodegradation of phenol and other organic and inorganic substances in wastewater from the industry is under consideration. Many studies show the biodegradation ability of pure or mixed cultures (Dursun & Tepe. 2005; Laowansiri et al., 2008; Celik et al., 2008). Pseudomonas, Bacillus, Escherichia, Acinetobacter, and Corynebacterium species demonstrates the potential for the industrial effluent treatments based on their ability to grow and utilize phenol present in waste water especially in petroleum refinery wastewater (Nwanyanwu & Abu, 2012; Zhenghui et al, 2016).
This study presents a possible alternative option to achieve bioremediation of phenol using the microbial isolation and adaptation. Isolation and characterization of naturally microorganisms from wastewater and activated granulated microorganisms are analysed to examine the degradation of refinery wastewaters by changes in phenol and other biological and chemical constituents.

2. MATERIALS AND METHODS

Wastewater samples were collected from the first and second aeration basin (bio based) of the refinery in the Republic of North Macedonia. Total of four sediments and water samples were collected from four different locations during autumn season. All samples were labelled and kept in sterile plastic bottle in room temperature. For the enrichment and isolation of the microbial culture (bacteria and yeasts) MSM (minimal salt medium, medium with minimal salt concentration) was used. The MSM contained; KH2PO4 0.5 g, K2HPO4 0.5 g, CaCl2 0.1 g, NaCl

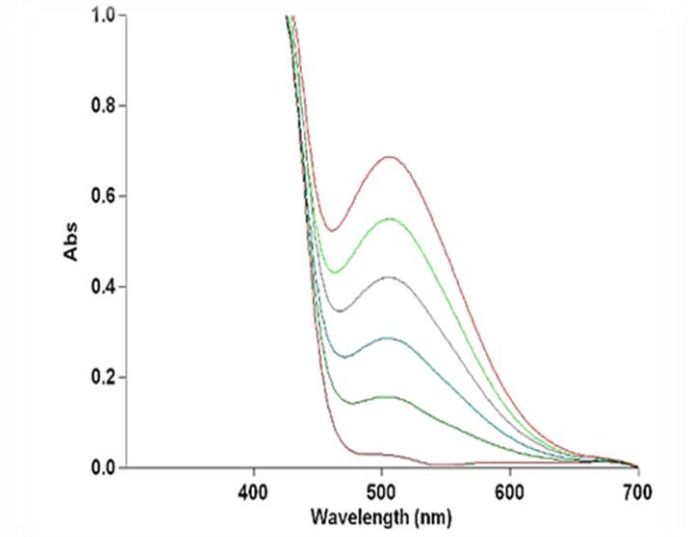
0.2 g, MgSO4_7H2O 0.5 g, MnSO4_7H2O 0.01 g, FeSO4_7H2O 0.01 g, NH4NO3 1.0 g per liter. Deionized, distilled water was used for the all experiments. The mineral substrate was supplemented with 100 mg /L^{-1} phenols. The bioreactor used in the experiment consists of an aerobic - segment for nitrification and anaerobic - for denitrification. The 250 ml culture was placed in a total volume of 2 L refinery waste water and aerated for 24 h. Air compressor enabled 1 mg / L - 1.5 mg / L dissolved oxygen. The same was performed in several phases of 24 h over a week, and the biological and chemical parameters for phenol concentration were followed at the beginning and at the end. Their determination was carried out according to standardized methods. 1500 ml of the supernatant were taken from the last stage of the anaerobic segment of the bioreactor for these analyses. 1 ml of the waste water sample was collected after 6 h and 24 h aeration, and the dilutions were made to 10^{-5}. TSA medium was used for the isolation of bacteria, YP and Rose Bengal Agar for the isolation of yeasts. The plates were incubated at 30 ° C. After Incubation and enrichment of bacteria and yeasts, they were selectively isolated until pure strain were obtained. Pure strains were inoculated on slag agar with paraffin and kept at a temperature of 4 ° C for further study.

Figure 2. (a) Laboratory Bioreactor (b) Phenol provided microbial cultures

(a) (b)

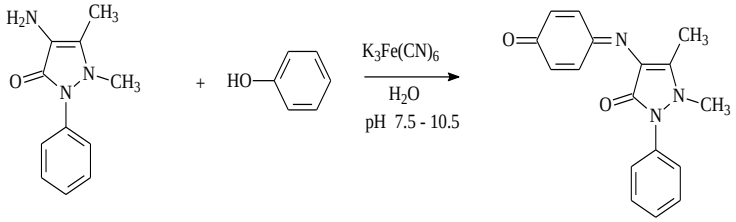
Before and after 24 h of aeration, the volume of biomass was monitored for one week. Analyses of the supernatant and the isolation selected of microorganisms were performed after each aerial. Microbial cultures were supplemented with 250 ml of MSM (mineral substrate and an appropriate concentration of phenol: 50, 100, 200, 300, 500, 800, 1000,1200 mg / L^{-1}. The resulting culture was placed in aeration and rotation to water bath shaker (72 h, 150 VM at a temperature of 28 ° C ± 1 ° C). The phenol concentration was determined by using 4-aminoantipyrine in the colorimetric assay (Spectrophotometric, Manual 4-AAP with Distillation), according to standard methods reported by the United States Environmental Protection Agency(EPA). Phenol was determined by buffering the sample to a pH of 10.0 and adding 4-aminoantipyrine to produce a yellow or amber coloured complex in the presence of ferricyanide ion (Figure 2).

Figure 3. UV-Vis spectra for calibration curve, Spectrophotometric, Manual 4-AAP with Distillation



The colour is intensified through extraction of the complex into chloroform. Measurement of this colour quantitatively determines the phenol concentration of the sample (Figure 3). (Chitra et al., 2018)

Figure 4. The Schematic reaction between phenol and 4 –aminoantipirine



The morphological properties of the isolated colonies were observed by optical microscopy. The typical physiological and biochemical characteristics of the phenol-degrading strains such as Gram's staining, motility was systematically performed according to standard manual methods.

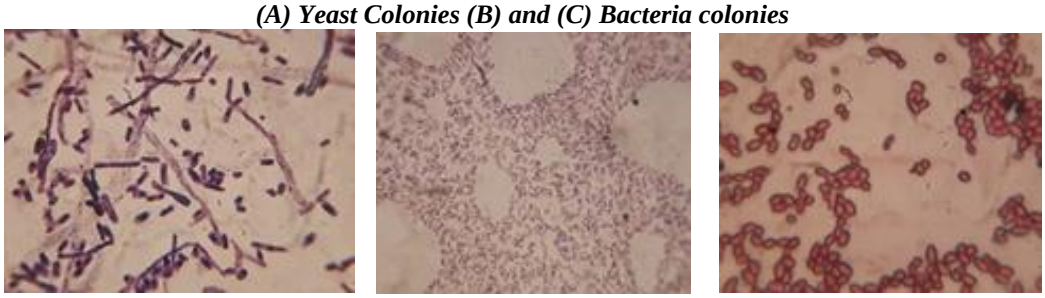
3. RESULTS AND DISCUSSIONS

The wastewater and sludge samples collected from an oil refinery effluent in North Macedonia were inoculated in the medium containing phenol for the enrichment and isolation of phenol-degrading bacteria and yeasts (Figure 5). incubation periods were repeated several times for the exact isolation and characterisation of microorganisms.

Figure 5. Isolated Bacteria and Yeast Samples

After three weeks of microbial enrichment from 300 mg/l of phenol isolates were obtained after 72 h growth on the Muller-Hinton and YP agar plates. Totally 9 isolates of 5 bacteria (BF), and 4 yeasts (KvF) were obtained after 72 h growth on the agar plates (Figure 6). All these strains utilized phenol as the sole carbon source 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)

The morphological properties of the isolated bacteria and yeast colonies were; Two Bacillus Gram+ bacteria, One Coccobacillus Gram+ bacteria and One Big-rod shaped Gram+ bacteria (Figure 7). Yeast colonies were with the typical physiological and biochemical characteristics of yeasts (Table 1).

Table 1. The Morphological Properties of Bacteria and Yeast Colonies

Isolates	Shape	Gram+,-
BF-1	Bacillus	+
BF-2	Bacillus	+
BF-3	Coccobacillus	+
BF-4	Bacillus	+
BF-5	Big Rod Bacillus	+
KvF-6	Yeast	/
KvF-7	Yeast	/
KvF-8	Yeast	/
KvF-9	Yeast	/

Final Strains were grown in batch cultures in 250-ml flasks containing 50 ml of MSM supplemented with phenol (1500 mg/liter) as the sole carbon source. The flasks were inoculated with 2 g/L and were incubated at 30°C in the dark on a rotary shaker (180 rpm) for 4 days (Figure 7).

Figure 7. Morphological and Microscopic Wives of Isolated and Adapted Bacteria and Yeast Samples

Bacillus subtilis sp. has been the best studied Gram-positive bacterium as a model for the many researches especially recent study of biofilm formation through a combination of genetic and biochemical approaches. *Bacillus subtilis sp.* is a widely adapted bacterial species, capable of growing within myriad environments including soil, plant roots and the animal gastrointestinal tracts. Capable of forming highly resistant dormant endospores in response to low food source and extreme environmental conditions allowing it to adapt and wide spread. Bacillus subtilis, is a Gram-positive, rod-shaped and catalase-positive bacterium (Figure 8). It has historically been classified as an obligate aerobe, though later evidence that it is a facultative anaerobe (Earl et al., 2008).
In this study, we reported the isolation of an enteric bacterial strain, *BIG FB-5*, which can degrade the phenol high concentrate as a sole source of carbon (Figure 8). Phenotypic profiling and sequence analysis identified the strain as *Bacillus subtilis*. There was also reported for the first time the degradation of phenol by other bacteria and yeast groups as well as their granules for further study of this research.(Table 2).

Figure 8. (A) Microscopic (B) Morphological Properties of Bacillus subtilis BIG FB-5

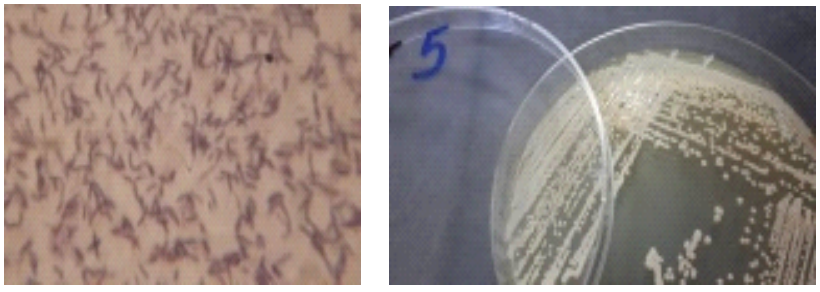


Table 2. The final Isolated Bacteria and Yeast Species

Sample	%	Family	Genus	Species
BF-1	99.9	Enterobacteriaceae	Leclercia	sp.
BF-2	90.1	Enterobacteriaceae	Raoultella	sp.
BF-3	99.9	Enterobacteriaceae	Citrobacter	sp.
BF-4	0	/	/	/
BF-5	81.9	Bacillaceae	Bacillus	subtilis
KvF-6	0	/	/	/
KvF-7	0	/	/	/
KvF-8	0	/	/	/
KvF-9	0	/	/	/

4. CONCLUSIONS

Different groups of microorganisms including bacteria, yeast and fungi can be found in natural environments contaminated with industrial hazardous wastes containing petroleum or plastic derivatives. Okereke et al. (2007) reported that few groups bacteria namely *Pseudomonas*, *Bacillus*, *Corynebacterium*, *Staphylococcus* and fungi groups; *Actinomycetes*, *Candida*, *Mucor*, *Rhizopus* and *Aspergillus* can survive in soil and water samples contaminated with residues of hydrocarbons and their derivatives.

In conclusion several colonies of bacteria and yeast capable of degrading petroleum wastewater and phenol was isolated from an oil refinery effluent and adapted to the degradation of high phenol concentration.

Initial experiments indicate that altered parameters in the effluent refinery water was decreasing unlike the influent.

The viability and resistance of microorganisms to high concentrations of phenol points to the possibility of their use in the environmentally and energy-efficient process of bioremediation.

The morphological properties of the isolates shoved that Bacteria types were Gram+ Bacillus type and Yeast isolates were with the typical yeast characteristics.

In conclusion we report the isolation and adaptation of a bacterial strain, BIG FB-1, which can resist and degrade the high phenol concentration. Phenotypic profiling and sequence analysis identified the strain as *Bacillus subtilis.*

It remains to carry out a detailed genetic analysis of all strains present in this study, as well as combining them with resistant strains of native microorganisms for obtaining adapted granules with greater ability for biodegradation of phenol as a highly toxic compound.

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