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A Study of the Ability of Pure and Mixed Cultures of *Staphylococcus warneri* and *Candida famata* Species to Biodegrade Naphthalene

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ABSTRACT

The biodegradation of naphthalene, a simple polycyclic aromatic hydrocarbon, has been of interest to researchers due to its toxic nature on living species. While some species of microorganisms are able to enhance naphthalene degradation in soils have been well documented, there is need to test the synergetic interactions of these organisms in order to improve biodegradation ability. In this work, pure and mixed cultures of *Staphylococcus warneri* (bacterium) and *Candida famata* (yeast), isolated by an enrichment method from a contaminated site in the Niger delta area of Nigeria were used to degrade naphthalene in liquid medium. The reactions were carried out in flasks placed in a shaking water bath at room temperature and 0.066 g for 14 days with samples withdrawn at two days interval for analysis. The dry biomass was analyzed by drying the layer containing the wet biomass in pre-weighed filter papers to constant weight, while the residual naphthalene concentration was obtained by spectrophotometric assay. Two replicates were used in determining both the dry biomass concentration and the residual naphthalene concentration. The results obtained showed that the pure culture of *C. famata* had an overall percentage degradation of 76.55 %, *S. warneri* 85.79 % while their mixed cultures had 92.28% after the 14 day experimental run. From the re-parameterized Monod Kinetic model, the maximum specific substrate consumption rate (q_s^{max}) obtained were 4.68, 1.63 and 0.72 $gg^{-1}day^{-1}$for *C. famata*, *S. warneri* and their mixed cultures respectively. The results indicate the possibility of using the organisms both in pure and mixed culture for biodegradation of naphthalene.

Keywords: Naphthalene, Biodegradation, Synergism, *Staphylococcus warneri*, *Candida famata*

1.0 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are compounds of environmental and human health concern since some them have been shown to be toxic, mutagenic, or even carcinogenic (Jacob *et al*., 1986; National Academy of Sciences, 1983). These Polycyclic aromatic hydrocarbons (PAHs) contain complex chemicals which include carbon and hydrogen with a fused ring structure, containing at least two benzene rings; and have effects on human health depending on the concentration, type and extent of exposure. Human exposure to PAHs may occur from emissions during the incomplete combustion of fossil fuels or due to accidental discharge into aquatic and terrestrial environments during the transportation, and disposal of petroleum products (Readman *et al*., 1982; Jacob *et al*., 1986). Naphthalene, the simplest PAH and one of the 16 PAHs classified as priority pollutants by the Environmental Protection Agency (EPA) of United States, has long been used as a model compound in PAH bioremediation studies. Despite its low solubility in water, it is frequently encountered in effluents in complex mixtures like petroleum fractions, creosote, and pharmaceutical wastes (Mueller *et al*., 1989). Systematic exposure to naphthalene and its derivatives has been shown to cause several diseases and disturbances to human metabolism (ATSDR, 1990).

Biodegradation of various hydrocarbons, which has been the subject of study for more than 40 years (Alexander, 1999) can serve to curb the environmental menace of this compound. The key component in biodegradation is the microorganisms which produce the enzymes involved in the degradative reactions leading to the elimination or detoxification of the chemical pollutant. Common naphthalene-degrading microbes include *Pseudomonas spp, Candida spp, Vibrio spp, Mycobacterium spp, Marinobacterspp, Staphylococcus spp* and *Sphingomona sspp* (Karl *et al*., 2000).

Although some species of microorganisms which are able to enhance naphthalene degradation in soils have been well documented, there is yet a need to test the synergetic interactions of these organisms. This is because many biodegradation processes have been found to require the cooperation of more than a single species (Reisfeld *et al*., 1972). These interactions may be necessary for the initial step in the conversion, a later phase of the transformation, or the mineralization of the compound. These various interactions represent several types of synergism, in which two or more species carry out a transformation that a pure species alone cannot perform or in which the process carried out by the multispecies mixture is more rapid than the sums of the rates of reactions effected by each of the separated species (Alexander, 1994). Thus, some reactions take place in mixtures of species but not in pure culture or take place more readily in multispecies associations.

The goal of biodegradation studies is to identify successful strains that can be used or applied in the remediation of contaminated sites. This study therefore aims to test the biodegrading abilities of *Staphylococcus warneri* and *Candida famata* in pure and mixed cultures on naphthalene, and also to estimate the substrate utilization parameters and the biomass growth parameters for both organisms.

2.0 MATERIALS AND METHODS

2.1 Microorganism

Pure cultures of *S. warneri* and *C. famata* were obtained by the enrichment method from a contaminated site in the Niger delta area of Nigeria.

2.2 Chemicals

Analytical grades of high purity (>99%) naphthalene which is the carbon and energy source as well as acetone, a proven solvent for the hydrocarbon, were obtained from the Department of Chemical Engineering, University of Lagos, Nigeria.

2.3 Equipment

The main equipment used in this project which were all available at the Department of Chemical Engineering and Central Laboratory, University of Lagos were:

- Shaking Water Bath, Grant OLS200 (Grant Instruments Cambridge Ltd.)
- Shenan LDZX-50FB (Vertical Heating Pressure Steam Sterilizer) Autoclave
- Jenway 6405 UV/VIS Spectrophotometer.

2.4 Stock solutions and media

The degradation experiments were performed using minimal salt medium (MSM) of composition (gl^{-1}): NaCl, 10.00; $MgSO_4.7H_2O$, 0.42; KCl, 0.29; KH_2PO_4, 0.83; $Na_2HPO_4.H_2O$, 1.25; $NaNO_3$, 0.42; which was prepared by dissolving the various salts in distilled water. The medium was also sterilized by autoclaving at 121^oC for 20 minutes.

A stock solution of naphthalene was prepared by dissolving it in acetone as its crystal structure cannot be dissolved directly in broth. The quantity of naphthalene for the experiment was taken from this solution into the experimental vessel and allowed to settle uniformly at the base and then waiting till all the acetone was removed by evaporation.

2.5 Inoculum Preparation

From the pure culture obtained, a loopfull was transferred to a 100 ml conical flask containing 50 ml minimal salt medium and 1% (v/v) of naphthalene as sole carbon source. This was done separately for each organism alongside a mixed culture loopfull of both organisms. These 3 setups were then incubated in a rotary shaker (0.066 g) for 32 hours at 30^oC and termed as the primary cultures.

The secondary cultures were also prepared by transferring 25 ml of the cultures to 25 ml fresh medium and incubating in 100 ml conical flasks in the rotary shaker (0.066 g) for 14 hours at 30^{o}C. Inoculums for the experimental runs were taken from the secondary cultures with the view that exponential growth phase was fully established.

2.6 Biodegradation Experiments

The biodegrading abilities of the microorganisms (*S. warneri* and *C. famata*) on naphthalene, in pure and mixed cultures were studied using 25 mg/ml of naphthalene into the 88 ml minimal salts medium contained in Erlenmeyer flasks whose mouths were covered with cotton wool. Each of the flasks (with the exception of the control) were inoculated with isolated strains of the pure and mixed cultures and incubated at 28 ^{o}C on a Water Bath Shaker at 0.066 g for 14 days with samples withdrawn at two days interval for analysis. Two replicates were used in determining both the dry biomass concentration and the residual naphthalene concentration.

2.7 Determination of the Time Course for Growth

The dry biomass concentration of the samples was used to monitor the bacterial growth. The 10 ml samples which were taken at two days interval were introduced into an equal volume of hexane, with two layers formed. One layer contained the naphthalene, with an initial concentration of 25 g/l, dissolved in the hexane while the other contained the wet biomass and minimal salt medium solution. The layer containing the wet biomass was carefully decanted and transferred to pre-weighed filter papers. The filter papers were then dried to constant weight in an oven at 60^{0}C with the averages of the weights recorded.

2.8 Determination of naphthalene degradation efficiency of the isolates

The efficiency of the isolated organisms to degrade naphthalene and its relation with the growth was studied by estimating the residual naphthalene after 2 days (48 h) interval. The supernatants (containing hexane with the dissolved naphthalene) obtained after decanting was extracted with a syringe and the naphthalene concentration monitored spectrophotometrically by taking absorbance at 540 nm against a blank of pure hexane (Zhang *et al.,* 2005). The quantitative determination of the concentration of constituents is as a result of the direct relationship between the amount of radiation which is emitted or absorbed, and the amount of element, molecule or compound present. To estimate the residual naphthalene, a standard graph was prepared using 5 to 25 mg/ml of naphthalene. The absorbance of the periodic samples was first obtained spetrophotometrically and then their respective hydrocarbon concentration estimates obtained from the plotted standard curve of concentration vs. absorbance.

3.0 RESULTS AND DISCUSSION

The results of the biodegrading abilities of the pure and mixed cultures in naphthalene are presented in Figures 1 – 3.

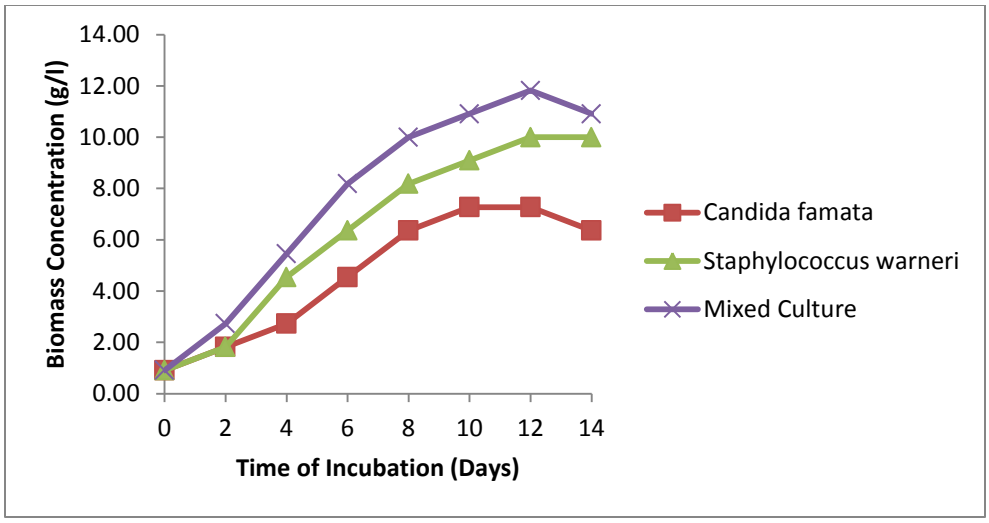

Figure 1: Biomass Growth Curve in Naphthalene at 28^{o}C in Minimal Salt Medium

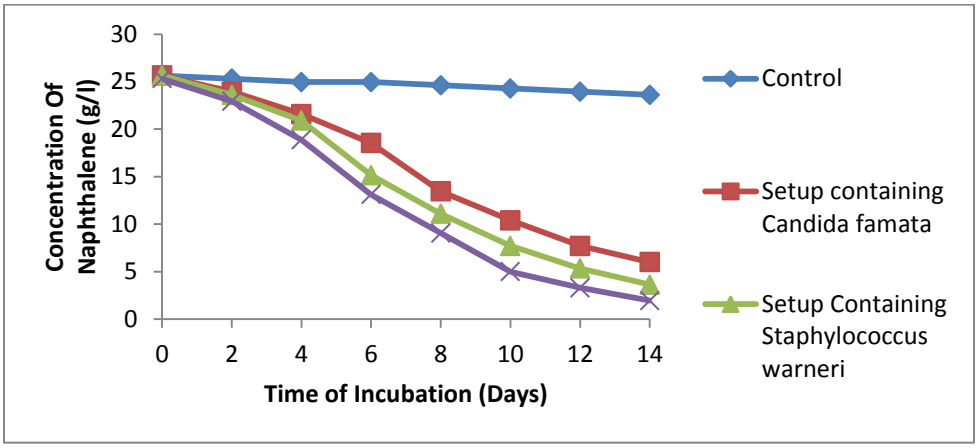

Figure 2: Residual Concentration of Naphthalene in Experimental Setups against time of Incubation

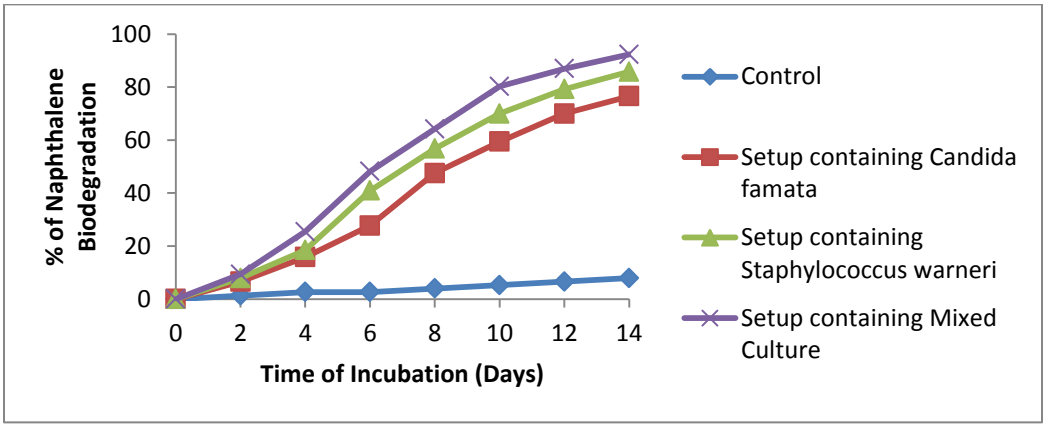

Figure 3: Percentage Biodegradation of Naphthalene by the pure and mixed cultures against time of incubation

The results obtained for the biomass concentration, residual substrate concentration, specific growth rate and specific substrate consumption rates are also presented in the Tables 1 – 3.

Table 1: Specific growth rate and substrate consumptions for pure C. famata culture

C. famata (Yeast)				
Time of Incubation (Days)	**Biomass Concentration X (g/l)**	**Substrate Concentration S (g/l)**	**Specific Growth rate μ (day^{-1})**	**Specific Substrate Consumption rate q_s ($gg^{-1}day^{-1}$)**
0	0.91	25.64	0.50	0.93
2	1.82	23.95	0.25	0.56
4	2.73	21.58	0.22	0.43
6	4.55	18.54	0.15	0.34
8	6.36	13.46	0.10	0.24
10	7.27	10.41	0.07	0.21
12	7.27	7.71	0.05	0.19
14	6.36	6.01	-	-

Table 2: Specific growth rate and substrate consumptions for the pure S. warneri culture

S. warneri (Bacterium)				
Time of Incubation (Days)	**Biomass Concentration X (g/l)**	**Substrate Concentration S (g/l)**	**Specific Growth rate μ (day^{-1})**	**Specific Substrate Consumption rate q_s ($gg^{-1}day^{-1}$)**
0	0.91	25.64	0.50	1.12
2	1.82	23.61	0.50	0.65
4	4.55	20.90	0.20	0.38
6	6.36	15.15	0.14	0.29
8	8.18	11.09	0.10	0.22
10	9.09	7.71	0.08	0.19
12	10.00	5.34	0.06	0.16
14	10.00	3.64	-	-

Table 3: Specific Growth rate and Substrate Consumptions for the mixed culture

MIXED CULTURE				
Time of Incubation (Days)	**Biomass Concentration X (g/l)**	**Substrate Concentration S (g/l)**	**Specific Growth rate μ (day^{-1})**	**Specific Substrate Consumption rate q_s ($gg^{-1}day^{-1}$)**
0	0.91	25.304	1.00	1.30
2	2.73	22.935	0.42	0.59
4	5.45	18.874	0.22	0.37
6	8.18	13.121	0.14	0.25
8	10.00	9.059	0.10	0.20
10	10.91	4.998	0.08	0.17
12	11.82	3.306	0.06	0.14
14	10.91	1.952	-	-

The growth profile of the two organisms and the mixed culture as shown in Figure 1 suggested that no lag phase was observed, this was due to the fact that the organisms were already acclimatized to the medium and were probably in the exponential growth phase.

The plots in Figure 1-3 and the results in Tables 1-3 show that naphthalene disappearance in the control system was slower than in the inoculated systems. This implies that though naphthalene sublimes naturally, its rate of disappearance was greatly increased by the organisms.

From Figure 3, it is observed that the natural sublimation of naphthalene in the control setup had an overall degradation of 7.92%, while the degradation by *C. famata* of and *S. warneri* was 76.55% and 85.79% respectively. Some synergism was observed because the mixed culture gave the highest degradation of 92.8% over a period of 14 days.

During the course of the first 10 days, a rapid and steady increase in biomass concentration was observed for all three set-ups and then the growth rate reduced. Similar decrease in the biomass concentration was reported for the growth of *Psuedomonas* strain on diesel oil after 5-days by some other researchers (Olanipekun *et al.* 2012) The decrease in the biomass concentration could be explained by three possible mechanisms namely, the production of some metabolites in the course of degradation may inhibit growth of the organisms, the possibility of nutrient depletion due to utilization and lastly cell adhesion to the glassware. Approximately 39% of cell adhesion to the glassware in the absence of surfactant was observed in some previous research work (Stelmack *et al.* 1999).

The two microbes, *C. famata* and *S. warneri* grew and were able to degrade the naphthalene under the conditions of the experiment. The results obtained are shown in Figures 1-3. It was observed that as the biomass increased the residual naphthalene concentration decreased, and the organisms both in pure and mixed culture grew quite effectively within the first six days. This stage which corresponds to the period with the high percentage of

naphthalene consumption, gave the following results of 27.72% by *C. famata*, 40.91% by *S. warneri* and 48.15% by the mixed culture. This result is corroborated by work done by (Plohl *et al*. 2002) and (Zhang *et al*. 2005) who observed that the first five days of incubation were said to be the most important for biodegradation. They observed that during this period majority of the compounds (especially n-alkanes) were degraded, and only a slight increase followed in the remaining days of incubation (Plohl *et al*. 2002).

It was observed that the specific growth rate, μ generally decreased as the specific rate of substrate consumption q_s, decreased. These results indicate that the growth rates of the organisms were a direct consequence of substrate consumption.

3.1 The Growth Kinetics of the Organisms on Naphthalene

The Monod type of model equation $q_s = q^{\max} s / k_s + s$ which (Layokun *et al*., (1987)) has been used extensively in waste treatment processes was linearized, hence the plot of $1/q_s$ against $1/S$ gave the Monod saturation constant k_s and the maximum specific substrate consumption rate $q^{\max}$ for the two organisms and their mixed culture. The values obtained are given in Table 4.

Table 4: Results of the kinetic model

ORGANISMS	q_s^{max} (gg-1day-1)	k_s (g/l)
Candida Famata (Yeast)	4.68	203.35
Staphylococcus Warneri (Bacterium)	1.63	55.40
Mixed Culture	0.72	15.09

S warneri had greater affinity for naphthalene than *C. famata*. The bacteria exhibited ability of high growth rate even at low level of the substrate concentration. This phenomena has also been observed by other researchers (Halden *et al*., 1999). The mixed culture exhibited a considerable greater affinity for the substrate, and this was corroborated in the value of k_s obtained.

The kinetic parameters for the mixed culture of *C. famata* and *S. warneri* showed an increased affinity of the organisms for the naphthalene. Therefore, the scavenging ability of the synergetic metabolism is higher than that of the pure cultures.

4.0 CONCLUSION

S. warneri and *C. famata* both in pure and mixed cultures showed potency and possibility of being used as agents for bioremediation.

S. warneri is better in terms of performance than *C. famata* in degrading naphthalene.

The mixed culture of *S. warneri* and *C.Famata* with overall Naphthalene degradation of 92.28% outperformed the pure cultures of *S. warneri* (85.79%) and *C. famata* (76.55%).

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