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Phenol Utilization by Yeasts Strains Isolated from Hydrocarbon Impacted Soil

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Abstract

The advent of modern technology and industrialization as well as environmental pollution of the natural resources by chemicals is of great concern. Most pollutants are released into the environment through industrial, agricultural and pharmaceutical wastes as mixtures of organic and inorganic pollutants. Pollutants released to the environment as a result of these activities include phenols, polycyclic aromatic hydrocarbons and heavy metals. The present study was carried out to assess phenol utilization potential of yeast strains isolated from hydrocarbon impacted soil. The yeasts isolated were identified as *Saprochaete* sp and *Cryptococcus* sp. The biodegradation phenol potential of *Saprochaete* sp. and *Cryptococcus* sp. was studied using mineral salt broth supplemented with phenol with concentration ranging from 0-1000 mg/l. Investigation showed that the yeasts were able to utilize almost all the introduced quantity (1000 mg/l) of phenol as sole source of carbon and energy within a period of 3 to14 days. The growth response of both yeasts on increased doses of phenol on mineral salt medium supplemented with different phenol concentrations ranging from 0-6mM showed decrease in the number of the yeasts as the concentrations of the phenol increased. The rate of phenol degradation for both organisms was the same at phenol concentration of 100 mg/l and 500 mg/l with degradation rate values of 0.083 mg/l.h and 0.250 mg/l.h respectively. *Saprochaete* sp and *Cryptococcus* sp present a great potential for the biodegradation of phenol and possibly of other related phenolic or aromatic compounds.

1. Introduction

Due to rapid industrialization and economic development, many pharmaceutical, petrochemical and chemical industries are releasing their effluents into natural ecosystem. Many of these aromatic compounds are toxic to biota and their presence in the aquatic and terrestrial habitats often have serious ecological consequences. Due to improper treatment of these materials soil and groundwater, widely contaminated have been toxicity affects living organisms seriously. Organic pollutants released into the environment as a result of these activities include phenol, polycyclic aromatic hydrocarbons. Among those pollutants, phenolic compounds pose a threat to the environment because of their toxicity and stability through bioaccumulation (Teixeira, Nildo de Abreu, Lidia & Fabiana, 2015).They are considered as one of the priority pollutants by the Environmental Protection Agencies (Wang, Zhong & Xu, 2012; Cravotta & Brady, 2015). Due to the increasing importance of petroleum for industrial development, petroleum refinery effluents have had a significant impact on the pollution of surface water systems and soil. The processing of crude oil in the refinery has led to the generation of large volumes of effluents (Nwanyanwu & Abu, 2012;

Coelho, Castro, Dezotti & Sant'Anna, 2006). The major pollutants accompanying these effluents include oil and grease, petroleum hydrocarbon, ammonia, sulphur, aromatic compounds and other unwanted constituents (Wake, 2005). Prominent among these aromatic compounds is phenol and its derivatives which contributed to a greater part of the toxic nature of refinery effluents. Sometimes they form complex compounds with metal ions, discharged from other industries, which are more carcinogenic in nature than the phenolic compounds (Nwanyanwu & Abu, 2012). Inspite of phenolic toxic properties, a number of micro-organisms can utilize phenol under aerobic conditions as sources of carbon and energy (Chen, Chen & Lin, 2006). Fungi are famous for their wide incidence and the outstanding capacity of degrading complex and inert natural products like lignin, chitin and cellulose. Fungi adapt more easily than bacteria and are capable of growing in extreme conditions, like nutrient deficiency, low pH, limited water supply, and others (Atagana, 2000; Supriya & Neehar, 2014) as well as their ability to survive in the presence of various xenobiotics than other microorganisms. To treat phenolic compounds, biological methods are preferable because this is economical, and there is a low possibility of the production of byproducts. Many studies support the biological treatment of pollutants. The microorganisms involved in phenol utilization include aerobes, including *Pseudomonas* sp. (Chitra, Sekaran, Padmavthi & Chandrakasan, 1995; Kim, Oh, Lee, Kim & Hong, 2002), *Alcaligenes* sp. (Hill, Milne & Nawrocki, 1996; Valenzuela, Bumann, Cespedes, Padila & Gonzalez, 1997), *Azotobacter* sp. (Li, Eberspacher, Wagner, Kuntzer, Lingens, 1991), *Rhodococcus* sp. (Apajalahti, & Salkinoja-Salonen, 1986; Oh & Han, 1997), *Phanerochaere* sp. (Perez *et al*., 1997; Larmar, Larsen & Kirt, 1990) and *Cryptococcus* sp. (Morsen & Rehm, 1987). Some yeast strains are reported to have the capability of utilizing phenol, Chandran and Das, (2012) isolated five species of yeasts including *Cryptococcus laurentii*, *Candida tropicalis*, *Rhodotorula mucilaginosa*, *Trichosporon asahii* and *Candida rugosa* from hydrocarbon-contaminated soil. Phalgune *et al*., (2013) explained the process of phenol biodegradation by the yeast *Candida tropicalis* NCIM 3556 in aqueous medium using DOSY NMR techniques. This test indicated that the phenol was completely degraded to carbon dioxide and water within approximately 20 h after incubation. Hassanshahian *et al*., (2012) described the ability of two different yeast strains, *Yarrowia lipolytica* PG-20 and PG-32, in decomposing aromatic hydrocarbons. Chandran and Das (2012) isolated *Candida tropicalis* from some oil contaminated soil that had high ability to produce biosurfactants capable of degrading diesel oil during ten days. These aerobes are more efficient at degrading toxic compounds because they grow faster than anaerobes and usually transform organic compounds to inorganic compounds (CO_2, H_2O). The ability of organisms to degradation of phenol and other toxicants is related to adaptation of the microorganisms to the compound of concern and adaptation is associated with synthesis of new enzymes capable of transformation of the toxicant to harmless substances. The resultant effect of biodegradation of phenol and other organic compounds is growth as the organic pollutants are used as the source of carbon and energy (Nwanyanwu & Abu, 2013). The aim of this study was to evaluate phenol utilization of yeast strains isolated from hydrocarbon impacted soil

The specific objectives of this study were (i) to isolate and identify fungi species from hydrocarbon impacted soil. (ii) evaluate the ability of the fungi species to degrade phenol. (iii) access the hydrocarbon tolerance of the fungi species.

2. Materials and Method

2.1. Sample Collection and Characterization

Petroleum products contaminated soil sample was collected from Orji mechanic Village in Owerri North, Imo State, Nigeria. The soil sample was collected using sterile metal cylindrical tools at a depth of 5 cm below the soil surface. The samples were transferred into sterile polyethylene bags and were immediately delivered to the laboratory for physicochemical analysis such as pH, total hydrocarbon (THC) was determined following the standard procedures (APHA, 1985). Calcium (Ca) and magnesium (Mg) were determined by EDTA titration while potassium (K) and sodium (Na) were determined by flame photometry. The percent organic matter (%OM) was calculated from the percent organic carbon (%OC) measured using Walker-Black (1934) wet oxidation method. Total nitrogen (TN) was determined using the modified Kjeldahl distillation methods (Juo, 1979),while Lead (Pb), Zinc (Zn), Copper (Cu) and Cadmium were determined by atomic absorption spectrophotometer (Perkins Elmer 3110) respectively.

2.2. Enumeration of Microorganisms

Ten (10) fold serial dilution of the sample was prepared by weighing out 1 g of the soil sample and suspended into 10 ml of sterile distilled water to form the stock. The mixture was stirred for 1 min in order to allow detachment of microbial cells from the soil particle. Using a sterile pipette, one militer (1.0 ml) of the solution was serial diluted in saline solution up to 10^{-8}. Thereafter, an aliquot 0.1 ml of the dilutions 10^{-7} were plated out in triplicates on potato dextrose agar (PDA) supplemented with 50 µg/ml of streptomycin antibiotic solution and 2 mM of phenol (modified from Vanishree *et al.,* 2014),nutrient agar supplemented with 50 µg/ml of ketoconazole, and nutrient agar plate without supplements respectively. The plates were then incubated at 30ºC for 48h for bacteria and total heterotrophic count. The colonies formed on each plate were enumerated and the average value was recorded as colony forming units per ml (cfu/g) of the soil sample.

2.3. Identification of Yeast Strains

Morphologically distinct yeast colonies that developed on potato dextrose agar supplemented with phenol were purified on nutrient agar and identified based on morphological, microscopy and biochemical (Assimilation and Fermentation) characteristics according to Campbell *et al.,* (2013).

2.4. Preparation of Inoculum

The phenol-utilizing yeast strains for the assay were grown in 100 ml of sterile nutrient broth media contained in 250 ml Erlenmeyer flasks. The flasks were incubated on a rotary shaker incubator operating at 150 rpm for 48 h at room temperature (28 ± 2^{o}C). Cells were harvested by centrifugation at 6000 rpm for 10 minutes. Harvested cells were washed twice in sterile water. The washed cells were resuspended in the same medium and the turbidity adjusted spectrophotometrically to give an optical density of 0.4 at absorbance of 600nm.

2.5. Growth on different concentrations of phenol amended mineral salt agar.

The mineral salt agar (NaCl, 10.0 g; $MgSO_4.7H_2O$, 0.42 g; KCl, 0.29 g; KH_2PO_4, 0.83g; Na_2HPO_4, 1.25 g; $NaNO_3$, 0.42 g; agar, 20 g; (Okpokwasili & Okorie, 1988)) was prepared.

The medium (50 ml) contained in 100ml Erlenmeyer flasks was supplemented with different concentrations ranging from 0-6.0 mM of phenol respectively. The stock solution was prepared by dissolving 0.094g 0f phenol in 100ml of sterile distilled water. Thereafter, the flasks were sterilized by autoclaving at 121ºC for 20min. after which the medium was poured into Petri dish plates and allowed to solidify at room temperature (28±2) ºC. Then one tenth (0.1ml) inoculum of the standardized organisms (OD_{600nm} 0.2) that was serially diluted to 10^{-3} was transferred onto the surface of the agar medium and spread plated. The plates were incubated at 30ºC for 72h after which the colonies that developed were enumerated as colony forming unit per milliliter (cfu/ml).

2.6. Biodegradation of phenol

2.6.1. Biodegradation assay

Degradation of phenol by the fungi species was carried out in sterile mineral salt medium (NaCl, 10.0 g; $MgSO_4.7H_2O$, 0.42 g; KCl, 0.29 g; KH_2PO_4, 0.83g; Na_2HPO_4, 1.25 g; $NaNO_3$, 0.42 g; agar, 20 g; (Okpokwasili and Okorie, 1988)), contained in 250ml Erlenmeyer flasks. The flasks were supplemented with aliquot of sterile phenol 2000 mg/l to bring the final phenol concentrations in the flasks to 100, 200, 500, 750 and 1000 mg/l. Thereafter, 2 ml of standardized (OD_{600nm} 0.4) cell suspension was added. The cultures were incubated at room temperature (28 ± 2^{o}C) on a rotary shaker operated at 150 rpm. Samples were withdrawn periodically (24h) to monitor the growth profile and utilization of phenol by the isolates. Phenol residue was determined in cell free supernatant via 4-aminoantipyrine technique.

2.7. Measurement of growth of pure cultures

Growth profile of the test organisms was monitored by using optical density. The optical densities (A_{600}) of the cultures were determined spectrophotometrically.

2.8. Assay for phenol residue

Phenol residue of the sample was determined using 4-aminoantipyrine method based on the procedure as described by Nwanyanwu and Abu, (2012). Culture samples were withdrawn and centrifuged 6000 rpm for 10min to remove the cells. Into 4ml of the sample supernatant contained in 20ml screw capped glass test tubes, 0.2 ml of 0.5N NH_4OH was added into it, followed by addition of 0.1 ml 2 %(w/v) 4- aminoantipyrine and 0.1 ml of 8 %(w/v) potassium ferricyanide [K_3Fe (CN) $_6$] and the contents thoroughly mixed. The absorbance of the resultant solution was determined spectophotometrically at 500nm. Phenol residue was then calculated by making reference to the standard curve.

2.9. Specific growth rate

The specific growth rates (μ, h^{-1}) were taken from $\ln(X/X_0)$ versus t-$t_0$plots for each initial phenol concentration. In each case, values were taken as the maximum slope in the respective plots (Nweke and Okpokwasili, 2014).

$$\ln\frac{\mathbf{X}}{\mathbf{X_0}} = \mu(\mathbf{t} - \mathbf{t_0})$$

2.10. Biodegradation rate

Phenol biodegradation rate (Qs, mg/l.h) was determined through the relationship of equation below (Nwanyanwu and Abu, 2012).

$$Q_s = \frac{d[ph]}{dt}$$

Where: [ph] denotes phenol concentration (mg/l), t denotes incubation time (h).

3. Results and Discussion

The physicochemical characteristics of the hydrocarbon impacted soil were shown in Table 1. The result showed that the pH of the soil is acidic with pH of 6.88; some of the metals in the soil include copper (0.50 kg), zinc (1.56 kg), lead (8.90 kg) and cadmium (3.70 kg). The total hydrocarbon content is 0.465 %. The microbiological analysis of the sample was shown in table 2. The total heterotrophic count is 3.3 x 10^6 cfu/g, the bacteria count is 1.6 x 10^5 cfu/g and phenol utilizing yeast is 0.6 x 10^5 cfu/g.

Table 1: physicochemical characteristics of the hydrocarbon impacted soil sample.

Parameter / unit	Value
pH	6.88
Phosphate (mg/kg)	18.64
Potassium (cmol/kg)	0.076
Calcium (cmol/kg)	0.986
Magnesium (cmol/kg)	0.372
Copper (kg)	0.50
Zinc (kg)	1.56
Lead (kg)	8.90
Cadmium (kg)	3.70
Total hydrocarbon content (%)	0.465
Total organic carbon content (%)	1.246
Total organic matter content (%)	2.148
Total nitrate content (%)	0.120

Table 2. Microbiological analysis of the hydrocarbon impacted soil

Microbial count	population (cfu/g)
Microbial heterotrophic	3.3 x 10^6
Bacteria	1.6 x 10^5
Phenol-utilizing yeast	0.6 x 10^5

The growth of the test isolates in solid culture using the mineral salt medium with varying concentrations of phenol (0 – 6.0mM) is shown in Figure 1. This is to assess the ability of the organisms to grow on mineral salt medium using phenol as the carbon source. Progressive

decrease in growth of the isolates was observed as concentration of the phenol increases. The highest growth was recorded at phenol concentration of 0.1mM at 1.07 x 10^2 CFU/ml for *Cryptococcus* sp and 1.10 x 10^2 CFU/ml for *Saprochaete* sp. The lowest growth was recorded at phenol concentration 0.5mM with 3.3 x 10^3 CFU/ml for *Cryptococcus* sp and 2.3 x 10^3 CFU/ml for *Saprochaete* sp. No growth was recorded at phenol concentrations of 1.0 to 6.0mM. Growth of the isolates at phenol concentrations of 0 to 0.5mM could be due to adaptation to the toxicity of the phenol as a result of the strains sources as well as utilization of phenol as carbon and energy sources. Though growth decreased progressively as the concentration of phenol increased in the media. Similar report was made by Aggelis and Lyberatos (2002) when they assayed for the removal of phenols in olive mill wastewaters using white-rot fungi.Abd El-Zaher *et al*., (2011), reported the effect of different concentrations of phenol on growth of some fungi isolated from contaminated soil, which shows that increase in phenol concentration decreases fungal growth.

Figures 2 and 3 showed degradation responses of the organisms over time at different initial phenol concentrations 100, 200, 500, 750 and 1000 mg/l.The phenol in the Mineral Salt Broth (MSB) was completely degraded by the organisms within 72h (3d) to 336h (14d) of incubation. One hundred milligram per litre (100 mg/l) and seven hundred and fifty milligram per liter (750 mg/l) of phenol were degraded completely within three days [3 d (72 h)] and ten days [10 d (240 h)] of incubation by both yeasts. For one thousand milligram per litre (1000 mg/l) of phenol, *Saprochaete* sp degraded the phenol within fourteen days [14 d (336 h)] of incubation, while *Cryptococcus sp* degraded the phenol within eleven days [11 d (264 h)] of incubation. The organisms were shown to possess innate ability to utilize phenol as source of carbon and energy, given that the organisms could grow and degrade phenol up to initial concentration of 1000 mg/l, they can be described as phenol resistant strains. They degraded low concentration of phenol relatively faster. The inhibition of microbial growth and biodegradation by phenol is a well-known phenomenon and has been reported in many organisms (Oboirien *et al*., 2005; Okpokwasili and Nweke, 2006; Agarry *et al*., 2010). The effect of different phenol concentrations on the growth of yeast strains showed that when the concentration of phenol was increased, the rate of degradation was decreased. Varma and Gaikwad (2009)) in their work investigated the degradation of phenol (200 mg L^{-1})by *Candida tropicalis* strain NCIM 3556, showing that this strain, in16 h, reached the maximum degradation (96.28%) and after48 h, the degradation was almost completed (99.88%).Liu *et al*. (2011) concluded that the yeast *T.montevideense* strain PHE1 well tolerated the toxicity of phenol concentrations above 2500 mg L^{-1}. *Saprochaete* sp degraded the phenol within fourteen days [14 d (336 h)] of incubation, while *Cryptococcus* sp degraded the phenol within eleven days [11 d (264 h)] of incubation, time-dependent degradation of organic compounds has been reported to be linked with concentration of the organic compound as observed by many authors (Colwell and Walker, 1977; Kotresha and Vidyasagar, 2008). This may be due to changes in the transport mechanism of the substrate across the cell membrane in response to high phenol concentration hence diminished capacity to catabolize phenol (Nwanyanwu and Abu, 2013). There was variation in degradation of the phenol by *Saprochaetes*p and *Cryptococcus* sp., the variation in degradation may be due to differences in cell wall components or poor induction of phenol hydroxylase systems, since phenol hydroxylase is known to be the major site for phenol inhibition (Leonard and Lindley. 1999).

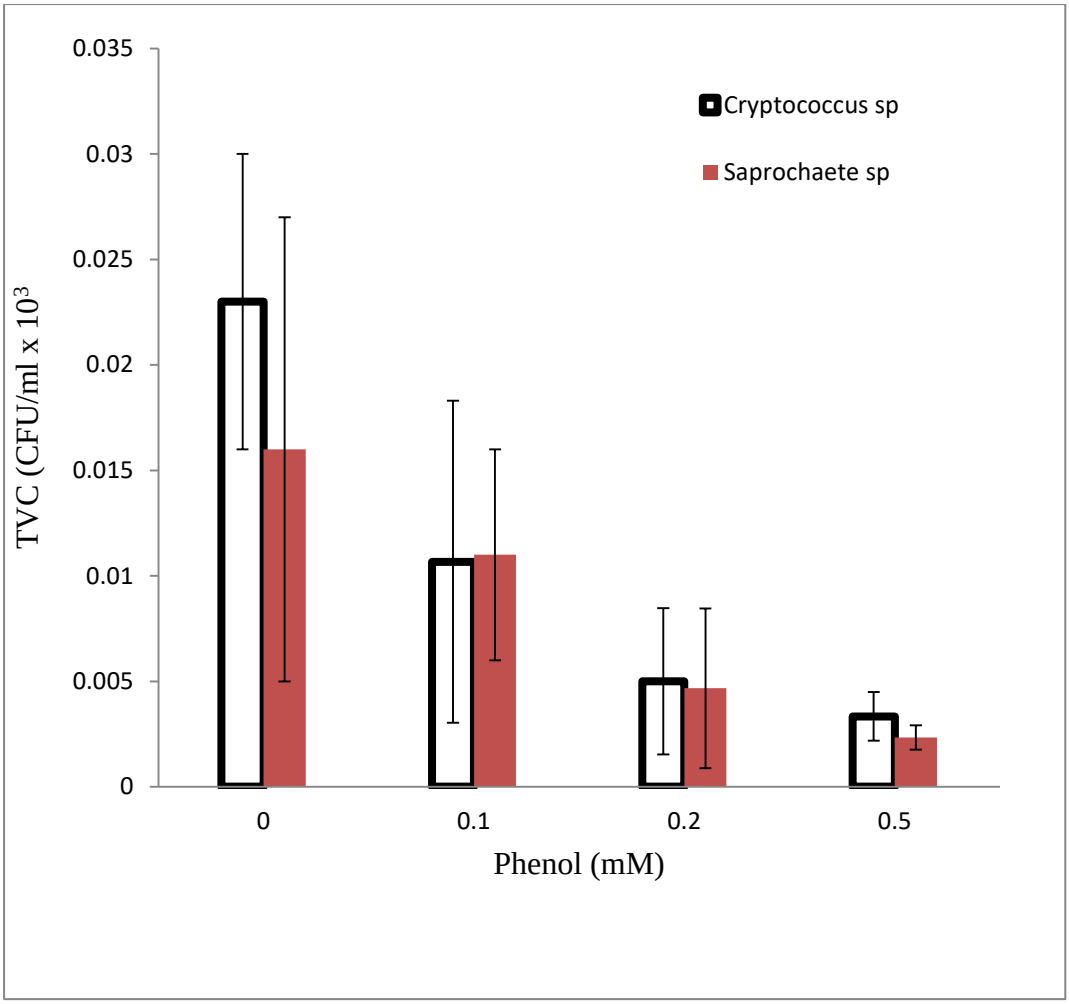

Figure 1: Growth response of the yeast to increased doses of phenol in mineral salt medium.

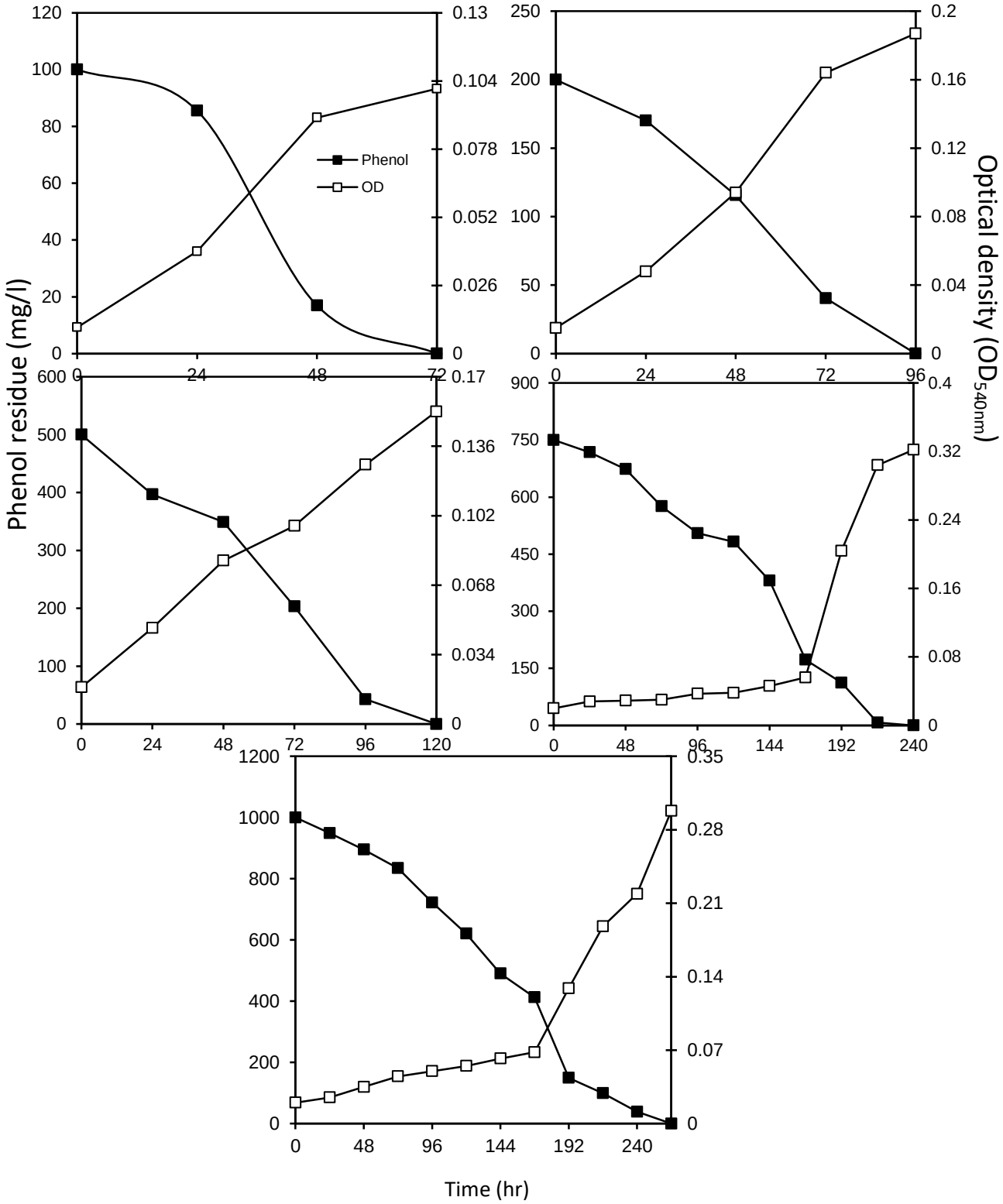

Figure 2: Growth profile and phenol degradation at different concentrations by *Cryptococcus* sp

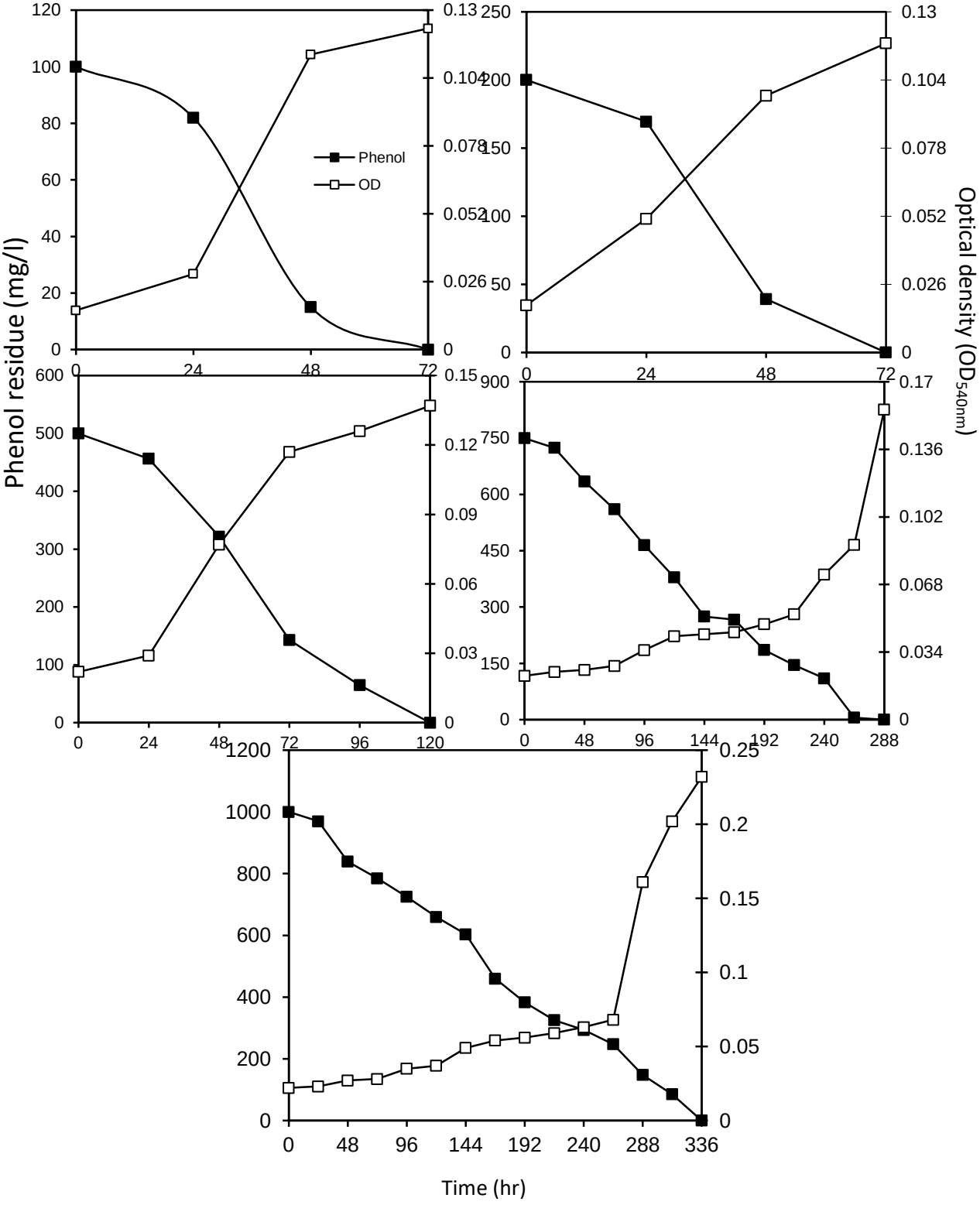

Figure 3: Growth profile and phenol degradation at different concentrations by *Saprochaete* sp

Table 3 showed the biodegradation responses of the isolates. The specific growth rate of both yeast decreased as the initial phenol concentrations were increased. The highest

specific growth rate was recorded at phenol concentration of 100mg/l with specific growth rate value of 0.030h^{-1} and 0.037h^{-1} for *Saprochaete* sp and *Cryptococcus* sp respectively; while the lowest specific growth rate was recorded at phenol concentration of 750ml/l and 1000mg/l with the same specific growth value of 0.009h^{-1} for *Cryptococcus* sp and 0. 005h^{-1} for *Saprochaete* sp.Both yeast recorded the highest degradation rate at phenol concentration of 500 mg/l with degradation value of 0.250 mg/l.h, and lowest degradation rate at phenol concentration of 100 mg/l with degradation value of 0.083 mg/l.h. *Saprochaete* sp and *Cryptococcus* sp recorded the highest specific degradation rate at phenol concentration of 500mg/l with 2.137mg/l.h.OD and 1.639mg/l.h.OD respectively, while the lowest specific degradation rate was recorded at phenol concentration of 1000 mg/l with 0.707mg/l.h.OD for *Saprochaete* sp and *Cryptococcus* sp recorded 0.582mg/l/h/OD at 750mg/l. The isolates showed decrease in the specific growth rate as the initial concentrations of the phenol increased. Similar result was shown by Santos and Linardi, (2001) in their work phenol degradation of yeast isolated from industrial effluent, reported that *Trichosporon* LE3 grew in the presence of up to 20mM phenol, but showed a reduction in specific growth rate at concentrations higher than 11mM, suggesting an inhibitory effect of phenol at these concentrations. According to Monod (1949), the higher the concentration of the growth-limiting substrate, the higher will be specific growth rate and the closer it will approach a maximum value. However, if this substrate shows inhibitory action, the continuous increment in specific growth rate should not happen, but rather a reduction in specific growth rate would occur, increasing substrate concentration (Haldane and Briggs, 1925).

Table 3: Biodegradation response of the yeast isolates

Phenol concentration (mg/l)	Organism	
	Cryptococcus sp	*Saprochaete* sp
	Specific growth rate (h^{-1})	
100	0.037	0.034
200	0.030	0.025
500	0.020	0.016
750	0.009	0.005
1000	0.009	0.005
	Biodegradation rate (mg/l.h)	
100	0.083	0.083
200	0.125	0.167
500	0.250	0.250
750	0.188	0.156
1000	0.227	0.164
	Specific biodegradation rate (mg/l.(h.OD))	
100	0.825	0.667
200	0.668	1.984
500	1.639	2.137
750	0.582	1.002
1000	0.764	0.707

The effect of different phenol concentrations on the growth of yeast showed that when the concentration of phenol was increased, the rate of degradation was decreased. Karimi and

Hassanshahian, (2016) reported that there was decrease in the rate of degradation with increase in initial phenol concentration, which is similar to result obtained in this study. The degradation rate and specific degradation rate in Table 3showed maximum phenol degradation at 500 mg/l by the isolates, this due to the fact that the phenol degrading enzymes activity is optimum at this concentration (Supriya1 and Neehar, 2014). Supriya1 and Neehar, (2014), reported maximum phenolic degradation at 300 mg/l by *Aspergillum niger*.

In conclusion, *Saprochaete* sp and *Cryptococcus* sp present a great potential for the biodegradation of phenol and possibly of other related phenolic or aromatic compounds. Such microorganisms can be further studied for use in industrial effluent treatment and decontamination of natural areas.

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