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Biodesulphurization Potentials of Microorganisms isolated from Coal, spent Refinery Catalyst and Hydrocarbon-contaminated Soil and Water Samples in a Tropical Ecosystem

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Abstract
The biodesulphurization (BDS) potentials of bacteria and yeasts isolated by enrichment in sulfur-free mineral salts medium (SFMSM) supplemented with 0.2 mM dibenzothiophene (DBT) from different samples in Nigeria were investigated. The rate of growth on DBT and conversion of the sulfite released from DBT to sulfate for growth was determined by measuring the optical density at 600 nm (OD_{600}) and 420 nm (OD_{420}) respectively, and the concentration of residual DBT in the culture medium after 72hrs determined by HPLC. All the isolates showed no appreciable increase in OD_{600} within the first 6 hrs; *Corynebacterium jeikeium* EMB112 and EMB111 have the highest and lowest OD_{600} of 1.3 and 0.39 respectively. Except for *C. jeikeium* EMB112 and *Candida valida* EMB911, sulfate production peaked at 72 hrs for all the isolates. However, there was a slight increase in sulfate concentration form 72 hrs (1.03 mM) to 144 hrs (1.08 mM) for strain EMB 112 while there is no change within the same time period for strain EMB911. Rate of DBT utilization (mM/day) ranged between 0.1035 (82.2%) and 0.1259 (100%). The utilization of DBT as a growth substrate by the organisms of the present study showed that they are potential candidates for BDS applications.

Key words: Spent refinery catalyst, dibenzothiophene, bacteria, yeast, biodesulphurization, coal.

Introduction

Sulphur dioxide (SO_2) emission through fossil fuel combustion is a global problem [1, 2]. SO_2 released into the atmosphere from the combustion of fossil fuels are the principal source of acid rain [3], can cause bronchial irritation and trigger asthma attack in susceptible individuals [4]. Potential health risks expand to a broader section of the public when the gas turns to particulate matter [4]. Long term exposure to combustion-related fine particulate air pollutant is an important environmental risk factor for cardio-pulmonary and lung cancer mortality [5].

The attendant health and environmental risks associated with SO_2 generated from the combustion of fossil fuels has continued to create a need for low sulfur fuels. Hence regulations guiding sulfur content of fossil fuels are becoming increasingly stringent. Prior to 2003, the US EPA proposed a reduction of the sulfur content of non-road diesel fuel from 3400 ppm to 500 ppm by 2007 [6]. Presently, the maximum allowable sulfur level for diesel in the USA is 15 ppm [7] and the European Union (EU) declared that the sulfur concentration in diesel fuels must be reduced <10 ppm by 2009 (European Union directive 2003/17/CE). These increasingly stringent requirements may eventually affect low sulfur fuels such as that produced in Nigeria. Hence the need for a means of desulphurization of the oil.

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Several gram positive and gram negative bacteria involved in BDS have been reported in literature [8, 9, 10, 11, 12], but studies investigating the BDS potentials of microorganisms isolated from countries producing low sulphur containing fossil fuels such as Nigeria are still very scarce. This is probably because reducing the sulphur content of fossil fuels in such countries is not considered a high priority focus of attention. Thus, evaluating the BDS potentials of locally isolated microbes in these countries is a necessary step, and an important prerequisite in the development of viable biological process for the production of affordable ultra-low sulfur fuels. This will enable such countries respond to the increasing global demand for ultra-low sulphur fuels.

Here we report the biodesulphurization potentials of some bacteria and yeast species isolated from heavy fuel oil, contaminated wastewater and soil samples collected from a heavy oil storage terminal located in Ijora, Lagos Southwest Nigeria, lignite and bituminous coals from Nigerian Coal Corporation, Enugu, eastern Nigeria, and spent platinum-molybdenum (Pt-Mo) catalyst from Port Harcourt Refinery, Nigeria.

Materials and Methods

DBT (98% purity) was obtained from Sigma-Aldrich (Milwaukee WI, USA). All other chemicals were obtained from reputable commercial sources in Nigeria and are of analytical grade. Lignite and bituminous coal were collected from Nigerian Coal Corporation, Enugu, Nigeria, Spent Platinum-Molybdenum catalyst was collected from Port Harcourt Refinery, Port Harcourt Nigeria, and heavy fuel oil contaminated soil and wastewater were collected from a heavy oil storage terminal in Ijora, Lagos Nigeria.

Bacteria Isolation

1 g of soil samples, pulverized coal or spent Pt-Mo catalyst, or 1 ml of wastewater were used as inocula in a selective enrichment process [3] for the isolation of DBT desulphurizing bacteria. Briefly, the samples were inoculated into 25 ml of sulphur-free mineral salts medium (SFMSM) (pH 7.0±0.2) containing K_2HPO_4 (4 g/l), $MgCl_2.6H_2O$ (0.2 g/l), $CaCl_2.2H_2O$ (0.001 g/l), $FeCl_3.6H_2O$ (0.001 g/l), NH_4Cl (2 g/l) with Glycerol (0.2%) as carbon and energy source. The medium was supplemented with 0.2 mM DBT prepared from a stock solution (2 mM) in absolute ethanol as sole source of sulphur. The stock was sterilized by filtration (Milllipore, 4.6 cm diameter, 0.2 µm pore size). 1.5 ml of the final enriched culture was spread on the surface of SFMSM supplemented with DBT and 1.5% agar-agar powder as a solidifying agent and the plates incubated at 30^{o}C. Colonies growing on the plates were purified and stored at 4^{o}C for biochemical characterization and BDS experiment.

Bacteria growth and sulphate production

The growth of the isolated organisms and the corresponding conversion of the sulfite released from the desulphurization of DBT to sulphate for growth were monitored by measuring the optical density (OD) at 600 nm (OD_{600}) and 420 nm (OD_{420}) respectively on a Jenway 6300 UV-Vis spectrophotometer. Each organism was cultured in 10 ml of SFMSM as described before. Duplicate cultures were selected for OD_{600} measurement at 0, 3, 6 and 24 h to determine the rate of growth on DBT. To quantify the amount of sulphate released from the metabolism of DBT, cell-free culture supernatants were reacted with $BaCl_2$ and HCl and the amount of sulphate precipitated was determined at 24, 72 and 144 h as the absorbance at 420 nm [13]. The quantity of sulphate (mM) was estimated from a standard curve plotted from the absorbance of 15 different standard solutions of sulphate with concentrations ranging from 0.1-4 mM. For each determination of OD_{600} and OD_{420}, the absorbance readings of uninoculated controls were also determined.

Quantification of residual DBT by HPLC

The concentration of residual DBT in the culture medium of nine isolates with the highest OD_{600} within 24 hr was determined by HPLC [14, 15, 16, 17] at the Central Science Laboratory, Obafemi Awolowo University, Ile-Ife, Nigeria. These include five isolates of *Acinetobacter* spp. (strains EMB221, EMB222, EMB72, EMB82, and EMB822), two isolates of *C. jeikeium* (EMB 112, EMB9131) and the two *Candida* spp. (strains EMB 52 and EMB 911). After 72 h of growth in 25 ml SFMSM with 0.38 mM DBT at 30^{o}C with agitation at 160 rpm, cell-free culture supernatants were acidified to pH 2.0 with 1M HCl and were extracted in n-hexane (1:1v/v) for 1 h. Uninoculated controls were similarly extracted and the n-hexane extracts analysed by HPLC (AKTA HPLC, Amersham Biosciences, Netherlands) equipped with a UV-Vis detector. Separation was carried out in a C_8 column at 25^{o}C with 70% acetonitrile as the mobile phase at a flow rate of 1.0 ml/min. Residual DBT and metabolites were detected at 280 nm. The concentration of residual DBT was estimated from a standard curve plotted with four standard solutions of DBT in 70% acetonitrile with concentrations ranging from 0.15 mM-1.0 mM.

Results and Discussion

Bacterial isolates

Eighteen distinct colonies growing on the SFMSM plates were selected and their identities determined by morphological and biochemical tests [18]. Four organisms each were isolated from the coal and contaminated soil samples; and five each from wastewater samples and the spent catalyst. Microscopy reveals that two of the organisms isolated from the spent catalyst were yeasts while all the other organisms are bacterial species. On the basis of their reactions in standard biochemical tests they were all identified as species of *Corynebacterium jeikeium* (5), *C. mycetoides* (1), *Acinetobacter baumannii* (8), A. *iwoffii* (1), *Bacillus mycoides* (1) and *Candida* (2) (Table 1). Previously, Omori et al. [8] and Baldi et al. [14] reported the desulphurization of DBT by a *Coryne-bacterium* sp. SY1 and *Rhodosporidium toruloides* DBVPG6662.

Table 1: Rate of Biodesulphurization (mM/day) of DBT by the isolated organisms determined by HPLC

Isolates	Source	Retention Time (Min)	% Desulphurization	Rate of Desulphurization (mM/day)
Control		5.46	ND	ND
Candida valida EMB 911	spent catalyst	no peak	100	0.1259
A. baumannii EMB 82	Lignite	no peak	100	0.1259
C. jeikeium EMB 9131	Spent catalyst	no peak	100	0.1259
A. baumannii EMB 72	Bituminous coal	5.67	99.6	0.1254
A. baumannii EMB 221	Soil	5.61	98.7	0.1242
C. krusei EMB 52	Wastewater	5.56	94.5	0.1190
A. iwoffii EMB 822	Lignite	5.61	92.9	0.1170
A. baumannii EMB 222	Soil	5.66	84.6	0.1065
C. jeikeium EMB 112	Wastewater	5.67	82.2	0.1035

ND: not determined

Growth on DBT and Sulphate Production

The time course of growth and sulphate production during growth on DBT are shown in Figures 1 and 2. All the isolated organisms showed similar growth patterns on DBT with no appreciable increase in OD_{600} within the first six hours of growth. Thereafter, the organisms showed varying patterns of growth: strain EMB112 was able to grow to the highest OD_{600} of 1.3 in 24 h while strain EMB111 was able to grow to an OD_{600} of 0.39, the lowest of the five strains. Except for

strain EMB112, the sulphate production profile of all the isolates also followed the same pattern with the highest concentration of detectable sulphate produced at 72 h followed by a reduction in concentration till 144 h (Fig. 2).

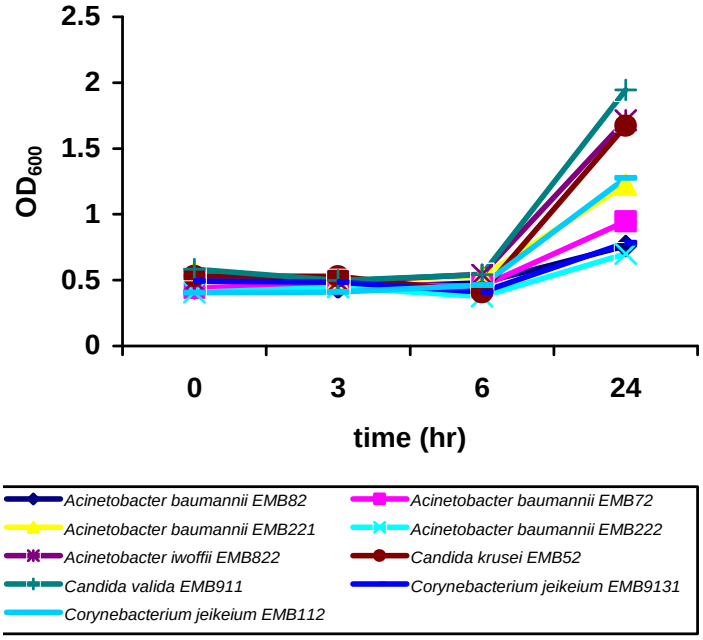

Fig. 1: Growth of the test organisms on DBT as determined by UV-visible spectrophotometry.

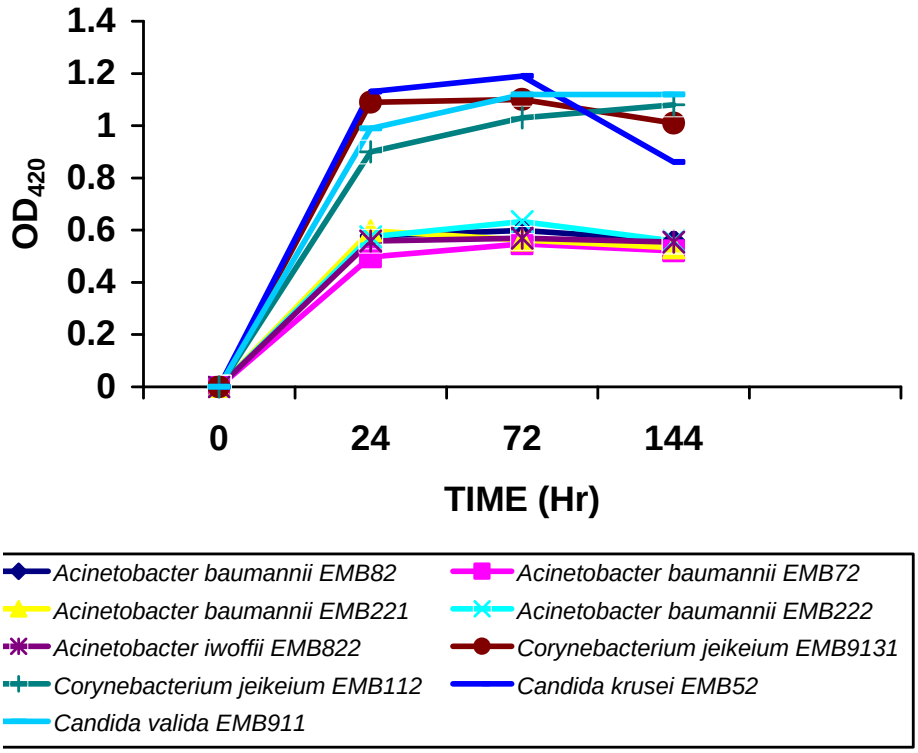

Fig. 2: Sulphate production by the test organisms during growth on DBT

The reduction in detectable sulphate may indicate a possible utilization of the sulphate for cell growth. However, there is a slight increase in sulphate concentration from 72 h (1.03 mM) to 144 h (1.08 mM) for strain EMB112, while there is no appreciable change in concentration within the same time period in the culture medium of EMB911. This may indicate a possible cessation in the utilization of sulfate produced from the metabolism of DBT for growth, or a potential for accumulation of free sulphate in the culture medium by these organisms. Though, strain EMB112 had the highest OD_{600} at 24 h, the accumulation of free sulphate in the growth medium is not favourable for BDS applications as free sulphate has been reported to repress BDS in various bacterial strains [4, 17].

On the basis of their high OD_{600} during their growth on DBT within 24 h, nine of the isolated organisms; *C. jeikeium* strains EMB112 and EMB9131, *Acinetobacter* spp strains EMB82, EMB72, EMB221, EMB22 and EMB822, and the *Candida* spp strains EMB911 and EMB 52 were selected for further analysis of residual DBT and metabolites by HPLC. Comparison of the HPLC chromatogram of the test organisms with that of uninoculated controls showed a decrease in concentration of DBT in the culture medium (Fig 3). The rate of utilization of DBT ranged from 0.104 mM/day to 0.13 mM/day (Table 1).

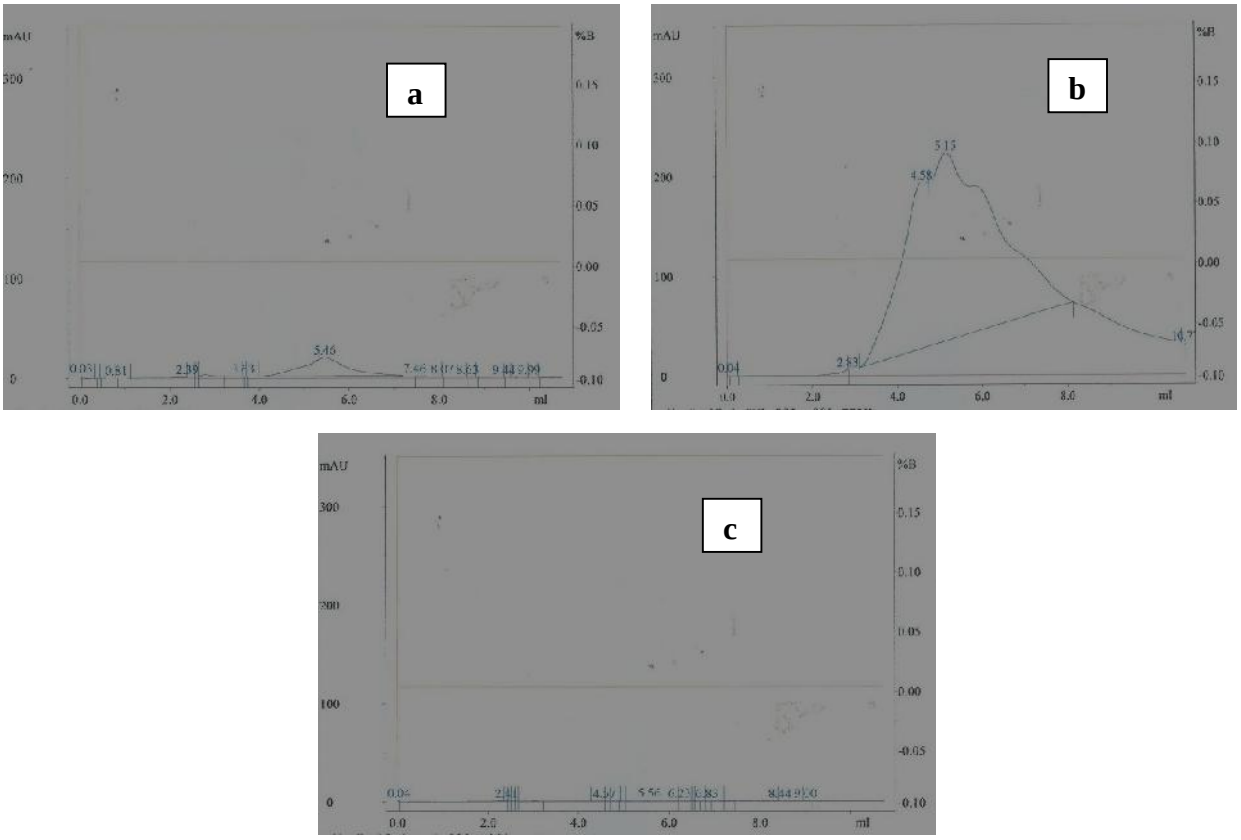

Fig. 3: Sample HPLC chromatogram of the n-hexane extracts of (a) uninoculated control with the pure compound (DBT) in 70% acetonitrile eluting at 5.46min (b) *Candida valida* EMB911 showing the metabolites produced eluting at 4.58min, 5.15min and 10.7min, and (c) *Candida krusei* EMB52 with no peak corresponding to produced metabolites.

No peak corresponding to residual DBT could be detected in the culture medium of *Candida valida* EMB911, *A. baumannii* EMB82 and *C. jeikeium* EMB9131 indicating a 100% desulphurization with a desulphurization rate of 0.1259 mM/day (Table 1). However, *C. jeikeium* EMB112 metabolized 82.2% of the DBT supplied as growth substrate with the least desulphurization rate of 0.11 mM/day. Three compounds considered to be metabolites of DBT detectable by HPLC were produced in high concentration in the culture medium of strain EMB911. The compounds eluted at 4.6 min, 5.2 min and 10.8 min respectively. However, little is known about the nature and fate of the metabolites resulting from sulphur removal from DBT by the other organisms reported in this study. No peak corresponding to products of DBT metabolism could be detected in the culture medium of these organisms.

It may probably be because the metabolites were produced in quantities too small for detection by the analytical method used in this study [14], or due to the liability of the products [3] or the existence of additional metabolic pathways for DBT metabolism in these organisms. Recently, Chen et al.[2] reported an extended 4S pathway of DBT desulfurization by *Mycobacterium* sp. ZD-19 involving the methoxylation of 2-HBP to 2-MBP, probably as a detoxification step for 2-HBP.

In conclusion, the microorganisms reported in this study are capable of growth on DBT in the presence of glycerol as a source of sulphur for growth and hence may be potential candidates for BDS applications. The detection of free sulphate in the culture medium of the organisms is taken as a further evidence of BDS activity. However, the potential accumulation of free sulphate in the growth medium of EMB112 and EMB911 is not favourable for BDS applications. Further studies will however be required to establish this and the suitability of these strains for BDS applications. Further studies are also needed to investigate the biochemical pathway of DBT metabolism by these strains and evaluate their potentials in BDS of crude oil.

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