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Biodesulfurization of Petro-diesel by a Novel Hydrocarbon Tolerable *Paenibacillus glucanolyticus* HN4

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Abstract **–** The presence of sulphur in petro-diesel has negative impact on engine performance and environment**.** In this work, a novel hydrocarbon tolerable *Paenibacillus glucanolyticus* HN4 denoted NCBI Gene Bank Accession No. MT645230; has been isolated from oil polluted sediment sample collected from Egyptian Red sea shoreline. Two-phase system enrichment medium containing 100 mg/l dibenzothiophene (DBT) dissolved in n-hexadecane (1/4 oil/water v/v) was used for selective enrichment and isolation of biodesulfurizing bacterium. HN4 desulfurized DBT as a model sulphur compound without affecting its hydrocarbon skeleton via the 4S-pathway producing 2-hydroxybiphenyl (2-HBP) as a dead end product. HN4 proved to be a hydrocarbon tolerant, biosurfactants(s) producer and endorsed a unique enzymatic system capable of desulfurizing both BTs and DBTs. Where, it desulfurized broad range of thiophenic compounds and expressed an efficient desulfurization activity against the recalcitrant alkylated DBTs. One-Factor-At-A-Time (OFAT) optimization technique illustrated approximately 90% biodesulfurization efficiency (%BDS) in an oil-water two-phase batch process at optimal operational conditions of; 120 h, 0.05 wt.% S-content model oil (DBT dissolved in n-hexadecane), 30^{o}C, pH7 and 1/1 oil/water phase ratio. HN4 decreased the sulphur content of a petro-diesel from 0.2 wt.% to 0.04 wt.%, in an 1/1 (v/v) oil-water two-phase batch BDS process, without affecting its calorific value. Consequently, that novel strain is recommended to be promising candidate for BDS as a complementary process for hydrodesulfurization technique in oil refinery.

Keywords**:** Biodesulfurization; Dibenzothiophene; Two-phase system; *Paenibacillus*, Hydrocarbon tolerance; Biosurfactants producer; Model oil; Petro-diesel oil.

1. Introduction

Incomplete combustion of fossil fuels causes emission of aromatic sulphur and nitrogen compounds, which upon oxidation lead to the aerosol of sulphuric and nitric acids the main contributors of acid rains [1,2]. Acid rains have many negative impacts on ecosystem and environment [3]. The NO_X emission can significantly increase up to 66%, corresponding to an increase in sulphur content of gasoline from 40 to 150 mg/L. About 73% of the produced SO_2 is from anthropogenic origin and is due to the combustion of petroleum and its derivatives [4]. The NO_X and CO_2 are the primary causes of "chemical smog" as well as "greenhouse gas" accumulation. Not only these, but, sulphur is also one of the main causes of emissions of particulate matter (PM), for example, approximately 2% sulphur in diesel fuel can be directly converted to PM emissions. All of those aforementioned harmful emissions add to the problem of climate change [2]. Although, the sulphur content increases with the boiling point during petroleum distillation, however, the middle distillates such as petro-diesel, may actually contain more sulphur than those of the higher boiling fractions as a result of decomposition of the higher molecular weight compounds during distillation [5]. Diesel exhaust is considered the most carcinogenic exhausts and accounts for approximately 25% of all smoke and soot in the atmosphere. It has been reported that relatively high concentration of SO_2 (> 100 ppm) expresses harmful effects to human respiratory system, where it can cause mortality within short time exposure to 400-500 ppm. Besides, very low concentrations of 1-2 ppm SO_2 would be enough to express sever damage to plant [2,6].

Hydrodesulfurization is the most widely applied method for removal of sulphur under high temperature and pressure in the presence of an expensive catalyst. In order, to achieve ultra-low sulphur diesel oil, more harsh conditions are required to remove the recalcitrant benzothiophenes and dibenzothiophenes [7]. That would increase operational and capital costs as well as more greenhouses gas (GHG) and H_2S emissions [8]. Biodesulfurization (BDS) proposed as a cost

effective and environmentally safe process for removal of recalcitrant sulphur compounds from petroleum fractions, under mild operational pressure and temperature [9]. However, the limited versatility of microorganisms towards the different sulphur compounds in real oil feed [10, 11], and their tolerance to solvents and hydrocarbons limit its applicability on industrial scale. Further, the bioavailability of organosulfur compounds in oil phase to microbial culture in aqueous phase is another obstacle that should be solved [9]. Surfactants can solve most of those obstacles [12].

The target of this work is to isolate and characterize novel hydrocarbon tolerable and biosurfactants(s) producer bacteria capable of selective biodesulfurization of different sulphur compounds in diesel oil without affecting its hydrocarbon skeleton and calorific value. The influence of various physical and chemical variables on batch oil-water two-phase BDS process has been also studied to maximize the BDS efficiency. Special emphasis on the effect of different oil/water phase ratios and different heterocyclic S-compounds was covered in this work. Finally, the BDS activity on a real petro-diesel oil feed was determined applying the predicted optimum conditions.

2. Experimental Work

Basal salts medium (BSM) supplemented with 100 mg/l DBT, were used for enrichment and isolation of an efficient biodesulfurizing bacterium from oil polluted sediment sample collected from Egyptian Red Sea shoreline [13]. Luria Bertani (LB) medium was used for inoculum preparation [13]. The concentration of bacterium used for inoculation was approximately 10^6 colony-forming units (CFU)/ml and the inoculation quantity of bacterium was 5% (v/v). One-factor-at-a-time technique (OFAT) was applied to study and optimize different factors affecting two-phase system-BDS process (i.e. DBT in n-hexadecane). Where, the factors (Table 1) studied in sequential experiments by varying the levels of one factor-at-a-time while fixing the other factors, in 100 ml working volume batch processes. The predicted optimum operating conditions were then applied to investigate the ability of the isolated bacterium to desulfurize a mixture of different sulphur compounds in a model oil; thiophene Th, benzothiophene BT, DBT, 4-methyl dibenzothiophene 4-MDBT and 4,6-dimethyl dibenzothiophene 4,6-DMDBT, each of 0.02 wt.% dissolved in n-hexadecane and a real petro-diesel sample with S-content of 0.2 wt.% and calorific value 45.7 MJ/kg. The desulfurized oil separated by centrifugation at 10,000 rpm for 10 min at 30°C. Gas chromatography-mass spectrometry (GC/MS) applied to elucidate the BDS pathway [14]. High performance liquid chromatographic analysis (HPLC) was used to follow up the BDS of sulphur compounds in model oil [15]. X-ray sulphur meter and calorific value tester were applied to determine the total sulphur content [16] and calorific value of the tested petro-diesel [17]. BDS was expressed as BDS (%) = $[(S_o - S)/S_o] \times 100$. Biosurfactant(s) production was detected in the cultures by measuring the surface tension of the medium using a DU-Nouy ringtype tensiometer [18].

Table 1: Studied experimental operating conditions and their ranges of operation.

Operating variable conditions	Operational range	Experimental conditions for maximum BDS-efficiency
Different incubation period	18 h – 168 h	pH7, 30°C, 150 rpm, ¼ (O/W), 0.1 wt.% DBT
Different DBT concentrations	0.01 – 0.2 wt.%	120 h, pH7, 30°C, 150 rpm, ¼ (O/W)
Different operating temperatures	15 – 40°C	120 h, pH7, 150 rpm, ¼ (O/W), 0.05 wt.% DBT
Different operating pH	4 - 9	120 h, 30°C, 150 rpm, ¼ (O/W), 0.05 wt.% DBT
Different oil/water (O/W) phase ratio	1/6 – 2/1 (v/v)	120 h, pH7, 30°C, 150 rpm, 0.05 wt.% DBT
Mixing speed	0 - 250 rpm	120 h, pH7, 30°C, 1/1 (O/W), 0.05 wt.% sulphur concentration
Different sulphur compounds	Th, BT, DBT, 4-MDBT & 4,6-DMDBT	120 h, pH7, 30°C, 150 rpm, 1/1 (O/W), 0.1 wt.% total sulphur concentration

3. Results and Discussion

3.1. Biodesulfurizing Bacterium Isolation and Identification

A Gram-positive, rod-shaped, motile, halotolerant, spore-forming, facultative anaerobic bacterium isolated from the collected oil polluted sediment sample for its ability to desulfurize DBT without attacking its hydrocarbon skeleton producing 2-hydroxybiphenyl (2-HBP) as a dead end product through the 4S-pathway (Fig. 1a). The 16S rDNA identified that isolate as *Paenibacillus glucanolyticus* HN4 (NCBI Gene Bank Accession No. MT645230) with % similarity of 99.24%; based on its phylogenetic tree (Fig. 1b). A thermophilic *Paenibacillus* sp. strain A11-2 has been reported as selective efficient biodesulfurizing bacterium for DBT and its alkylated derivatives [19]. Wang et al. [20] reported the isolation of two thermophilic *Paenibacillus* sp. from soil sample for their ability to selectively desulfurize DBT into 2-HBP. *Paenibacillus glucanolyticus* sp. strain T7-AHV was isolated by Ghafari et al. [21] from polluted marine environment for bioremediation of oil hydrocarbons polluted soil. As far to our knowledge; *Paenibacillus glucanolyticus* strain HN4 is first to be isolated for selective BDS, under mesophilic conditions.

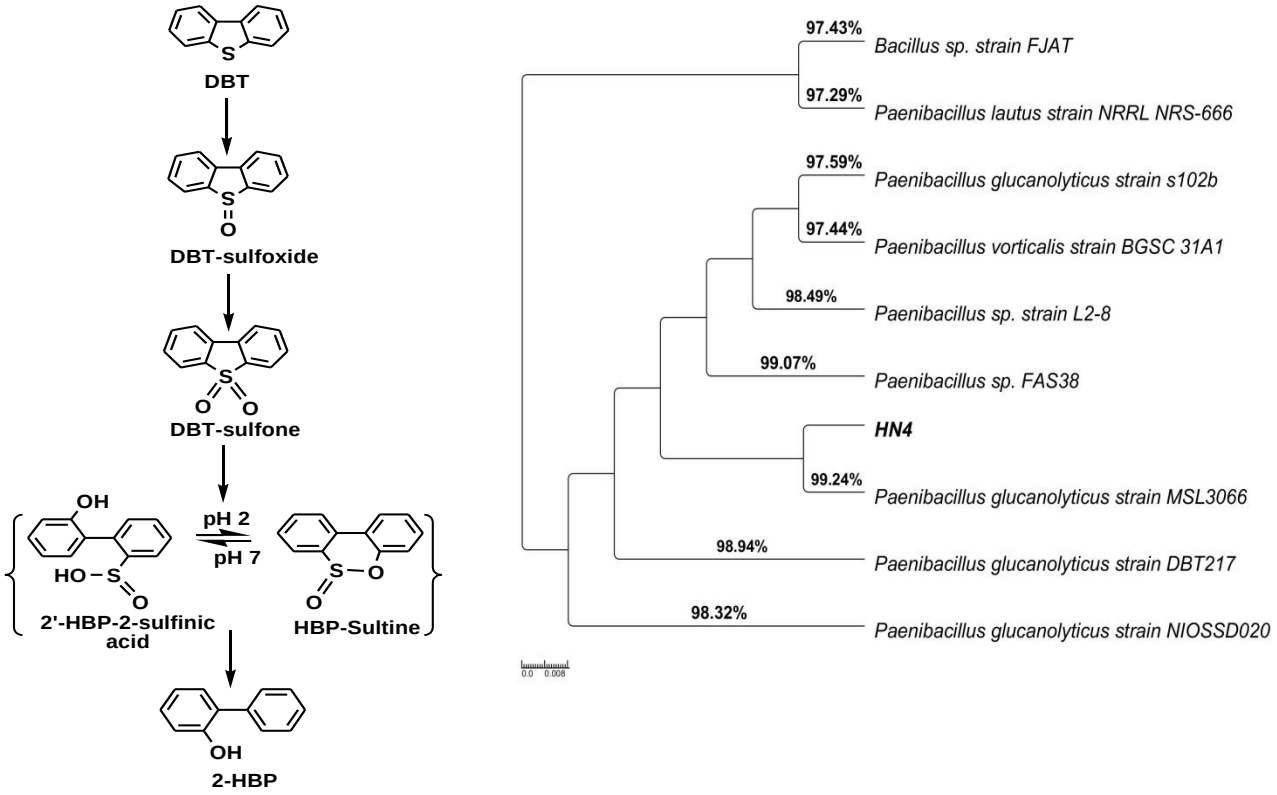

Fig. 1a: Elucidated DBT-BDS pathway by HN4, 1b: phylogenetic tree constructed by neighbour joining method of 16S rDNA gene for HN4 and closely related bacteria.

3.2. Optimization of two-Phase System BDS Batch Process

The two-phase BDS system (0.1 wt.% DBT in n-hexadecane ¼ O/w) using *P. glucanolyticus* strain HN4 found to follow the first order kinetic model equation (R^2 0.9397) with rate constant of 0.0076 h^{-1} and $t_{1/2}$ 91.2 h. HN4 observed to adhere to the oil-water interface and produced a strong stable emulsion. That is an indirect evidence for the production of biosurfactants(s) [22]. HN4 proved by GC/MS analysis to produce 2'-HBP-2-sulfinic acid which is reported to be starting material for novel surfactants [23, 24]. From the practical point of you to save time and energy the optimum operating time set as 120 h, because it thereafter entered in the stationary phase (Fig. 2). The optimum operating conditions found to be

0.05 wt.% DBT, pH7, 30ºC, where it recorded approximately 80% BDS efficiency (Fig. 3-4). However, it sharply decreased at higher sulphur concentrations (≥ 0.2 wt.% Fig. 3a). The changes in pH would affect the enzymes' 3-D and the electrical charge on the substrate, which consequently would retard or inhibit the substrate binding to the sites of enzymes. Thus, inhibits the enzymatic catalytic activities [25]. In addition, the 4S-pathway enzymes are be active at pH around neutrality (pH 6-8) [26]. *Paenibacillus validus* (strain PD2) isolated from oil polluted soil for its ability to desulfurize DBT via 4S-pathway producing 2-HBP [25]. Response surface methodology (RSM) based on 3^3 Box Behnken design was employed to optimize DBT-BDS batch process [25], which revealed relatively similar optimum conditions to those of HN4, recording; 0.41 mM DBT, pH 6.92 and 31.23°C.

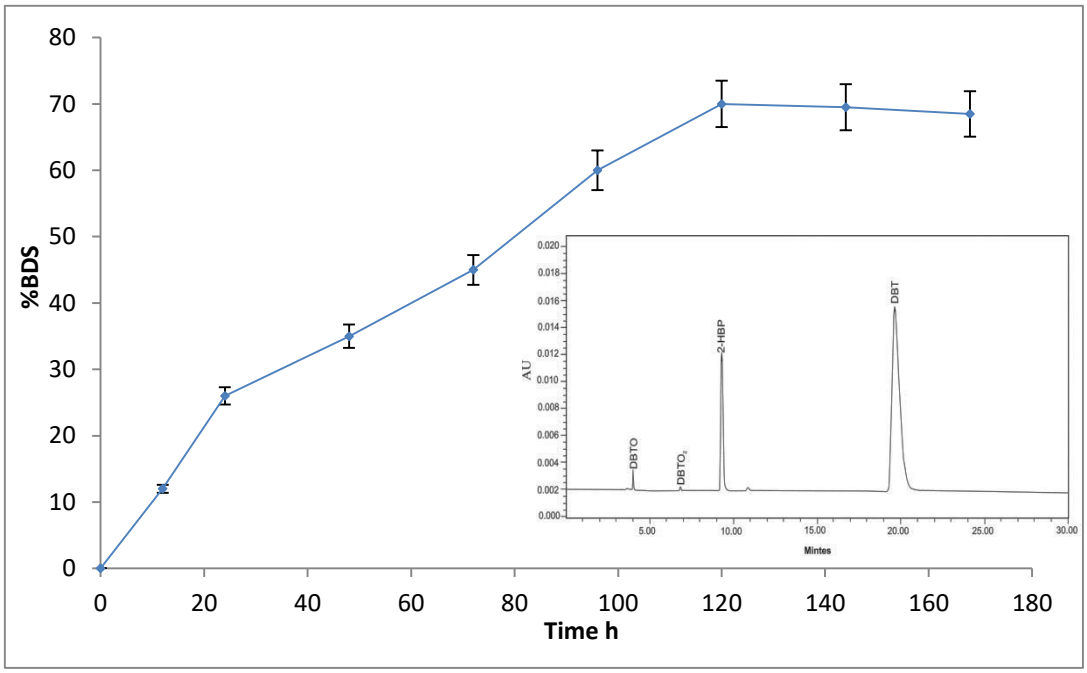

Fig. 2: Effect of operating time.

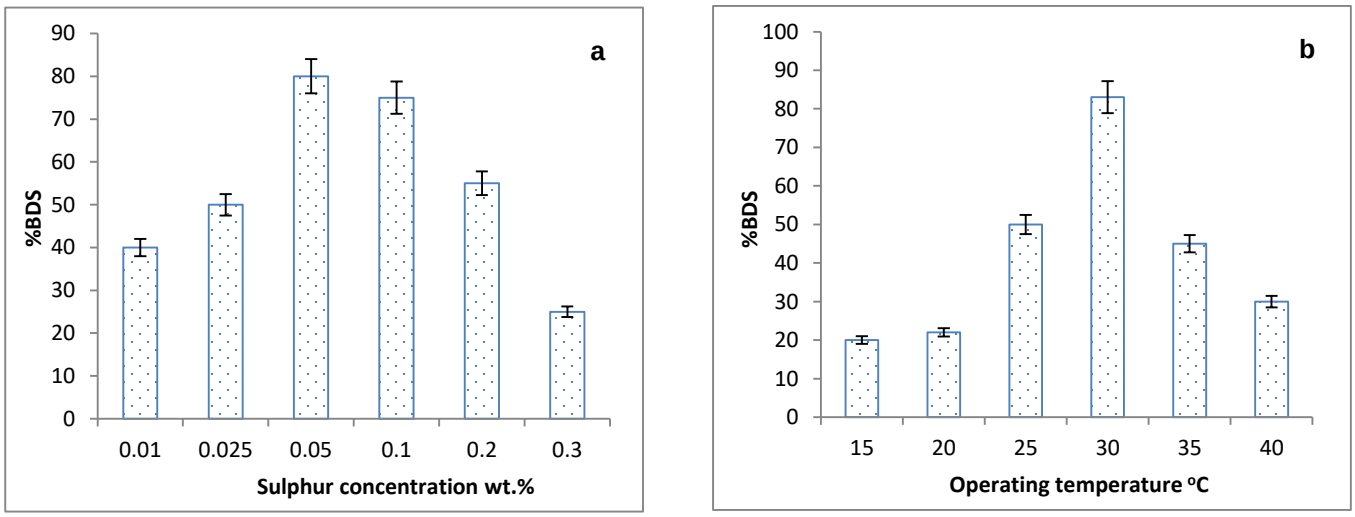

Fig. 3: Effect of sulphur concentration (a) and operating temperature (b).

It can be concluded from data illustrated in Fig. 4b that *P. glucanolyticus* strain HN4 is tolerable to high concentrations of hydrocarbons, since the BDS efficiency increased with the increase of O/W phase ratio recording approximately 90% at 1/1 O/W. Nevertheless, the BDS efficiency decreased by approximately 22%; at higher O/W phase ratio. From the economic point of view, as long as there is no significant difference in BDS efficiency within mixing rate (150 - 200 rpm, Fig. 5a), thus 150 rpm would be considered the optimum. Nevertheless, at higher mixing rate (> 200 rpm), the turbulence increase is harmful to bacteria, causing cell rupture and the mass-transfer limiting the BDS-process would be turned to reaction control [15].

It is worth to mention that there was no significant difference in BDS efficiency within the concentration range 0.05-0.1%, thus, higher concentration was applied in examining the effect of different S-compounds on the BDS efficiency to be more mimic with the industrial real oil feed in the refining process. It is obvious from Fig. 5b that HN4 has a wide versatility to desulfurize different S-compounds. That suggests that HN4 may have a novel enzymatic system for BDS of heterocyclic sulphur-containing compounds. However the BDS efficiency relatively decreased with the increase of molecular weight, aromatic ring numbers and substitutions. This might be due to the steric hindrance effect that might have prevented the microbial attack and/or the apparent competitive inhibition of substrates [27, 28]. Similar observation was reported by Konishi et al. [29] using thermophilic *Paenibacillus* strains in two-phase system of DBTs dissolved in n-tetradecane. Konishi et al. [30] reported also similar results to our findings, where, *Paenibacillus* sp. strain A11-2 selectively desulfurized BT more efficiently than DBT. To our knowledge few biodesulfurizing native strains are capable of desulfurizing both BTs and DBTs, for example; *Rhodococcus* sp. KT462, *Rhodococcus erythropolis* KA2-5-1, *Gordonia* sp. HS126-4 N and SC-10, *Sphingomonas subarctica* T7b, *Mycobacterium goodii* X7B and *Bacillus subtilis* WU-S2B [11, 31-36]. It is also known that high molecular weight alkylated DBTs, especially those alkylated at the 4- and/or 6-position are difficult to be biodesulfurized by most of the biodesulfurizing microorganisms [1]. Thus, *Paenibacillus glucanolyticus* strain HN4 with its efficient BDS capacities towards such alkylated-DBTs, is considered as a real and efficient added-value to the BDS field, since such thiophenic compounds can represent up to 70% of the total organic S-compounds in crude oil and its fractions.

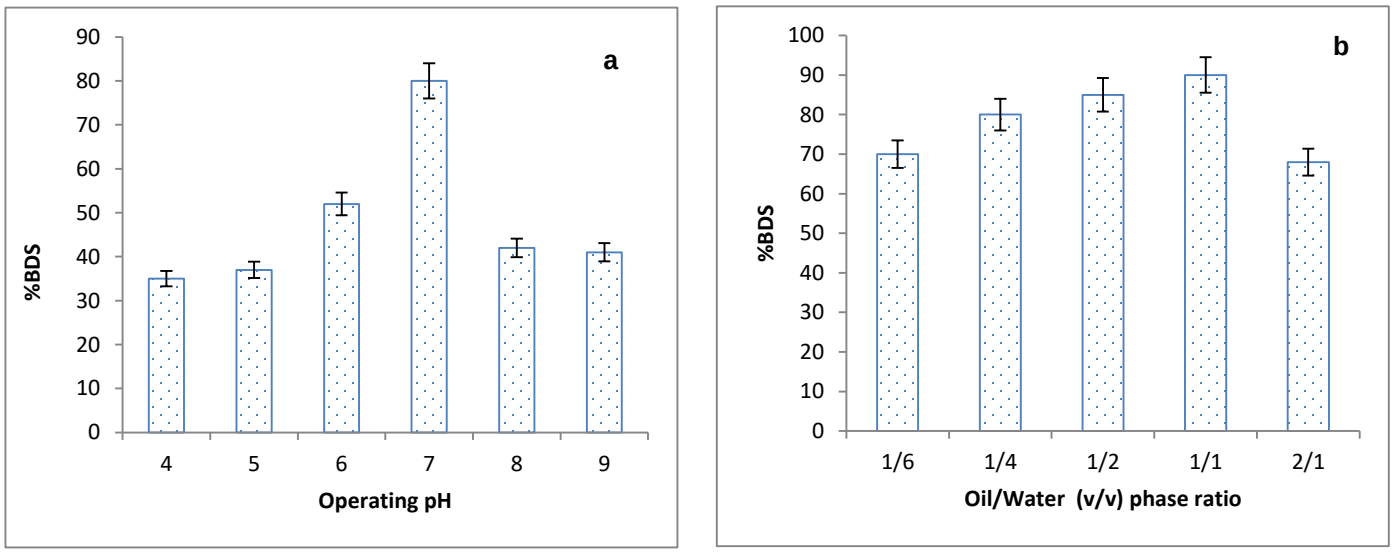

Fig. 4: Effect of operating pH (a) and oil/water phase ratio (b).

The surface tension of the culture was decreased by 45%. That proved the production of biosurfactants(s) which would enhance the bioavailability of the S-compounds and mass transfer, consequently enhance the BDS rate. Similar observation was reported by Agarwal and Sharma [37] and Amin et al. [38] and explained the increase of BDS rate by the amended solubility of the hydrophobic substrates in water via the development of reversed micelles which enriched the surface area of oil-water interface, that consequently, overcome the mass transfer limitation and amended the transport of DBT into the microbial cells found in the aqueous phase. Production of biosurfactants(s) would explain the tolerance of HN4 for high DBT and hydrocarbon concentrations, as biosurfactants limit their toxicity and inhibitory effects.

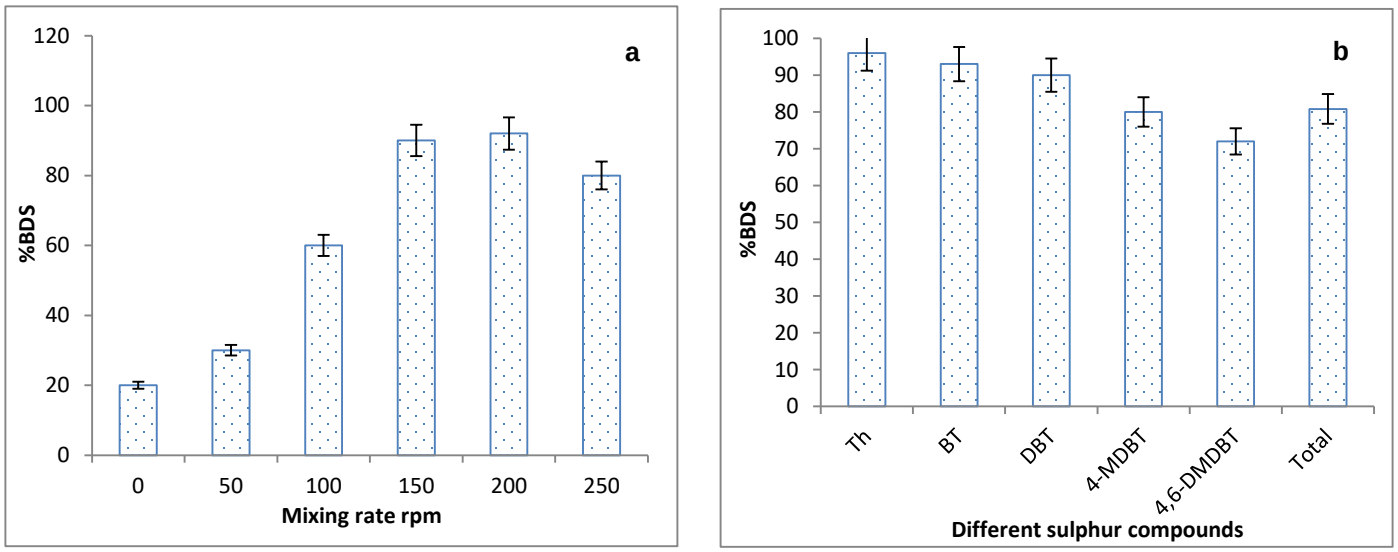

Fig. 5: Effect of mixing (a) and different sulphur compounds (b).

3.3. Biodesulfurization of Real Petro-diesel Oil Feed

Upon the application of HN4 in a two-phase (1/1 v/v) batch BDS of a real petro-diesel oil, the sulphur content decreased from 0.2 wt.% to 0.04 wt.% without any negative effect on the calorific value of the oil feed stock, recording 45.69 MJ/kg. Thermophilic *Paenibacillus* strains were previously reported for an efficient BDS of hydrodesulfurized light gas oil at 50°C [29]. The recorded efficient BDS activity by HN4 with its high hydrocarbon tolerance and wide versatility proved that; the oil polluted sediment sample used for isolation would serve as a bioavailable source for C, N, and S needed for indigenous microbial growth and enriched the enzymatic system of such micro-flora. Consequently, the presence and abundance of microbial strains with high tolerance to oil recalcitrant components in such oil contaminated habitats would support the isolation of microbial biocatalyst with an activated and enriched enzymatic system, that tolerate high concentrations of toxic hydrocarbons and biodesulfurize different recalcitrant S-compounds, in addition to the low molecular weight S-compounds. That was confirmed by the absence of lag phase (Fig.2), indicating the well endurance and adaptation of *Paenibacillus glucanolyticus* strain HN4, taking into consideration that DBT was used for enriching medium used HN4 isolation, which enhances the selectivity of the isolate.

4. Conclusion

The ability of the newly isolated HN4 to release sulphur from DBT without attacking its hydrocarbon skeleton, recommends its application for BDS of oil and its fractions without affecting its calorific value. Besides, its high hydrocarbon tolerance and biosurfactants(s) production increase its chance for industrial application. The broad versatility of HN4 to desulfurize different heterocyclic S-compounds, especially the recalcitrant alkylated DBTs, adds

to its favourability for industrial application as a complementary for hydrodesulfurization process. Nevertheless, further work is required on its enzymatic and genetic system involved in the BDS of DBTs and BTs.

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