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RESEARCH ARTICLE

Biodesulfurization of refractory sulfur compounds in petro-diesel by a novel hydrocarbon tolerable strain *Paenibacillus glucanolyticus* HN4

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Abstract

One of the main precursors of air pollution and acid rains is the presence of the recalcitrant thiophenic compounds, for example dibenzothiophene (DBT) and its derivatives in transportation fuels. In an attempt to achieve the worldwide regulations of ultra-low sulfur transportation fuels without affecting its hydrocarbon skeleton, a biphasic medium containing 100 mg/L DBT dissolved in n-hexadecane (1/4 oil/water v/v) used for enrichment and isolation of selective biodesulfurizing bacterium from an oil-polluted sediment sample collected from Egyptian Red Sea shoreline. The isolated bacterium is facultative anaerobe, motile, spore-former, and mesophile. It is genetically identified as *Paenibacillus glucanolyticus* strain HN4 (NCBI Gene Bank Accession No. MT645230). HN4 desulfurized DBT as a model of the recalcitrant thiophenic compounds without affecting its hydrocarbon skeleton via the 4S-pathway producing 2-hydroxybiphenyl (2-HBP) as a dead end product. HN4 substantiated to be a hydrocarbon tolerant, biosurfactants(s) producer, and endorsed unique enzymatic system capable of desulfurizing broad range of thiophenic compounds and expressed an efficient desulfurization activity against the recalcitrant alkylated DBTs. As far our knowledge, it is the first reported BDS study using *P. glucanolyticus*. Statistical optimization based on One-Factor-At-A-Time (OFAT) technique and response surface methodology (RSM) applied for elucidation of mathematical model correlations describing and optimizing the effect of different physicochemical parameters on batch biphasic BDS process. That illustrated an approximate increase in BDS efficiency by 1.34 fold and recorded 94% sulfur removal in biphasic batch process at optimum operation conditions of 120 h, 0.14 wt% S-content model oil (DBT dissolved in n-hexadecane), 33.5 °C, pH7 and 1/1 oil/water phase ratio, and 147 rpm. Resting cells of HN4 in a biphasic reactor (1/1 v/v) decreased the sulfur content of a refractory thiophenic model oil (thiophene, benzothiophene, DBT, and alkylated DBT dissolved in n-hexadecane) from 0.14 to 0.027 wt%, and petro-diesel from 0.2 to 0.04 wt%, within 120 h, keeping the calorific value of the treated fuel intact. Consequently, that novel strain could be recommended as a promising candidate for BDS as complementary to hydrodesulfurization process in oil refinery.

Keywords Selective biocatalytic desulfurization · Dibenzothiophene · Two-phase system · Oil tolerance · Biosurfactants producer · Model and real feed oil

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Introduction

Incomplete combustion of fossil fuels causes the emission of aromatic sulfur and nitrogen compounds, which upon oxidation, lead to the aerosol of sulfuric and nitric acids, the main contributors of acid rains (Sadare et al. 2017; Chen et al. 2018). Acid rains have many negative impacts on the ecosystem and environment (Martínez et al. 2016). The NO_X emission can significantly increase up to 66%, corresponding to an

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increase in gasoline sulfur content from 40 to 150 mg/L. About 73% of the produced SO_2 is from the anthropogenic origin and is due to the combustion of petroleum and its derivatives (Porto et al. 2018). The NO_X and CO_2 are the primary causes of "chemical smog" and "greenhouse gas (GHG)" accumulation. Not only this but sulfur is also one of the main causes of emissions of particulate matter (PM); for example approximately 2% sulfur in diesel fuel can be directly converted to PM emissions. All of those aforementioned harmful emissions add to the problem of climate change (Sadare et al. 2017). Although the sulfur content increases with the boiling point during petroleum distillation, however, the middle distillates such as petro-diesel, usually contain more sulfur than those of the higher boiling fractions as a result of the decomposition of the higher molecular weight compounds during distillation (Mohebali and Ball 2016). Diesel exhaust is considered the most carcinogenic exhaust and accounts for approximately 25% of all smoke and soot in the atmosphere. It has been reported that a relatively high concentration of SO_2 (> 100 ppm) expresses harmful effects to the human respiratory system, where it can cause mortality within short time exposure to 400–500 ppm. Besides, SO_2 reported to negatively affect the phytosynthesis process, reduce the carotenoid and chlorophyll contents, and increase the water use efficiency in plants (Lee et al. 2017).

Hydrodesulfurization is the most widely applied method for the removal of sulfur from oil feeds under high temperature and pressure in the presence of an expensive catalyst (Tao et al. 2011; Heidarinasab et al. 2016). In order to achieve ultra-low sulfur diesel oil, more harsh conditions of higher H_2 concentrations, more expensive catalysts, more elevated temperatures (≥ 425 °C), and higher pressure (≥ 250 psi) are required to remove the recalcitrant benzothiophene (BT), dibenzothiophene (DBT), and their alkylated derivatives (Abid et al. 2019). That would increase the operational and capital costs as well as the GHG and H_2S emissions (Lateef et al. 2019). Biodesulfurization (BDS) is proposed as a cost-effective and environmentally safe process for removal of recalcitrant sulfur compounds from petroleum fractions, under mild pressures and temperatures, especially upon applying whole-cell biocatalysis instead of the expensive enzymes (Canales et al. 2018). However, it has some obstacles limiting its applicability on an industrial scale: (1) BDS of oil feeds is a biphasic system, in which the oils containing organic solvents, such as; alkanes, alkenes, alcohols, and other aromatic hydrocarbons, which are usually toxic to microorganisms (Tao et al. 2006, 2011; Soleimani et al. 2007; Davoodi-Dehaghani et al. 2010). (2) Besides, the limited versatility of microorganisms towards the different sulfur compounds in the real oil feed (Chen et al. 2019). (3) Further, the bioavailability of organosulfur compounds in the oil phase to microbial culture in the aqueous phase is another obstacle that should be solved (Canales et al. 2018). Dinamarca et al. (2014) suggested the application of surfactants to overcome the mass transfer limitations and mitigate the solvent toxicity.

This work aims to isolate and characterize novel hydrocarbon tolerable and biosurfactants producer bacteria capable of selective biodesulfurization of different sulfur compounds in diesel oil. Response surface methodology (RSM) based on one-factor at-a-time technique was applied to (1) statistically investigate the influence of various physical and chemical factors on batch oil-water-two-phase BDS process to maximize the BDS efficiency and (2) modelling mathematical correlations describing the effect of the studied physicochemical parameters on the BDS efficiency. Particular emphasis on the effect of different oil/water phase ratios and different heterocyclic S-compounds was covered in this work to prove the broad versatility of the isolated novel strain. Finally, the BDS activity on a real petro-diesel oil feed was determined by applying the predicted optimum conditions.

Materials and methods

Chemicals

Thiophene (Th), benzothiophene (BT), dibenzothiophene (DBT), 4-methyldibenzothiophene (4-MDBT), and 4,6-dimethydibenzothiophene (4,6-DMDBT) (99% purity) are products of Merck, Germany. Glycerol (99% purity) was purchased from Honeywell, Germany. Yeast extract was obtained from Oxoid, UK. n-Hexadecane (99.8% purity) was purchased from Sigma-Aldrich, Spain. Acetonitrile (Ace) and Water (W) of HPLC grade were obtained from POCH, Poland. All other chemicals were of analytical grade, commercially available, and used without further purification.

Isolation of selective dibenzothiophene biodesulfurizing bacteria

This was done according to Nassar et al. (2013). Basal salt medium BSM (Caro et al. 2008) supplemented with 100 mg/L DBT dissolved in n-hexadecane (i.e., biphasic system 1/4 v/v) (Acero et al. 2003) was used for enrichment and isolation of an efficient biodesulfurizing bacterium from oil-polluted sediment sample collected from Egyptian Red Sea shoreline. That was briefly done by six successive transfers of 1 mL of the enriched mixed culture into fresh DBT/n-hexadecane/BSM batch of 250 mL Erlenmeyer flasks with a working volume of 100 mL each of 7-days incubation period at 30 °C and 150 rpm. Serial dilutions (10^{-1}) of each transfer on BSM-agar plates supplemented with 100 mg/L DBT were done and then incubated for 7 days at 30 °C. The bacterial colony that tolerated and survived after those six cycles was picked and purified on fresh Luria Bertani-agar plate for further use.

The isolated bacterium was genetically identified based on its 16S rDNA sequence (Query sequence) analysis on NCBI server http://www.ncbi.nlm.nih.org, using BLAST tool of the National Center for Biotechnology Information (NCBI) http://blast.ncbi.nlm.nih.gov/blast.cgi. DNA extraction, PCR purification, and amplification have been done in National Research Center, Egypt. DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene was performed using DreamTaq Green PCR Master Mix (Thermo Scientific, USA) , as directed by manufacturer's instructions. Two sets of universal primer, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACG ACTT-3′) were used and amplified about 1500 bp of the 16S rDNA region. The amplification conditions were as follows: 94 °C (5 min), followed by 35 cycles at 94 °C (1 min 30 s), 50 °C (45 s), and 72 °C (1 min 30 s). The amplified product was purified using the Qiaquick gel extraction kit (Qiagen, Germany) and sequenced on Applied Bio-systems automated DNA sequencer, model ABI 3730XL DNA Analyser (Applied Bio-systems, USA; service provided by Macrogen Inc., South Korea). The phylogenetic tree was constructed by the neighbour-joining method applying the Jukes-Cantor model, for calculating the genetic distances, and finally, MEGA7 software was used to draw the tree.

Growing and resting cell inoculum preparation

Luria Bertani medium is a complex medium used for maintenance and inoculum preparation (Caro et al. 2008). Cells of bacterial isolates were inoculated in pH 7 Luria Bertani broth medium, and then incubated at 30 °C for 24 h in a shaking incubator at 150 rpm. Cells were harvested by centrifugation at 5000 rpm (4752 g) for 15 min then washed twice with BSM. For growing cells preparation, the biomass was re-suspended in fresh, sterilized BSM free of DBT. While for resting cells inoculum preparation, the biomass was re-suspended in BSM supplemented with 100 mg/L DBT and then incubated for the late exponential phase in a shaking incubator set at 150 rpm and 30 °C. The biomass was then harvested as listed before by centrifugation, washed and, re-suspended in BSM free of DBT.

Batch biphasic BDS process

One-factor-at-a-time (OFAT) technique was applied to study and optimize different factors affecting biphasic system-BDS process (i.e. DBT in n-hexadecane/BSM). Where, the factors (Table 1) were studied in sequential experiments by varying the levels of OFAT while fixing the other factors, in 250 mL Erlenmeyer flasks of 100 mL working volume batch processes. 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). The predicted optimum operating conditions were applied to investigate the ability of the isolated bacterium to desulfurize: (1) a mixture of different sulfur 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 0.028 wt% dissolved in n-hexadecane, and (2) a real petro-diesel sample with S-content of 0.2 wt% and calorific value 45.7 MJ/kg. The desulfurized oil was then separated at the prescribed operation time by centrifugation at 15,000 rpm (22,640 g) for 10 min at 30 °C (Folsom et al. 1999). All the experiments and measurements were done in triplicate, and the arithmetic averages were used throughout the data analysis and calculations.

Table 1 Studied experimental operating conditions and their ranges of operation

Operating variable	Operational range	Experimental conditions for reaching maximum BDS-efficiency
Different incubation period	0–168 h	pH7, 30 °C, 150 rpm, 1/4 (O/W), 0.1 wt% DBT (5.43 M), growing cells
BDS efficiency in aqueous system	120 h	pH7, 30 °C, 150 rpm, 0.1 wt% DBT (5.43 M DBT dissolved in ethanol), growing cells
Different DBT concentrations	0.01–0.2 wt%	120 h, pH7, 30 °C, 150 rpm, 1/4 (O/W), resting cells
Different operating temperatures	15–40 °C	120 h, pH7, 150 rpm, 1/4 (O/W), 0.05 wt% DBT (2.72 M), resting cells
Different operating pH	4–9	120 h, 30 °C, 150 rpm, 1/4 (O/W), 0.05 wt% DBT (2.72 M), resting cells
Different oil/water (O/W) phase ratio	1/6–2/1 (v/v)	120 h, pH7, 30 °C, 150 rpm, 0.05 wt% DBT (2.72 M), resting cells
Mixing speed	0–250 rpm	120 h, pH7, 30 °C, 1/1 (O/W), 0.05 wt% DBT (2.72 M), resting cells
Different sulfur compounds	Th, BT, DBT, 4-MDBT, and 4,6-DMDBT	120 h, pH7, 30 °C, 147 rpm, 1/1 (O/W), 0.14 wt% total sulfur concentration, resting cells
Petro-diesel oil feed	0.2 wt% sulfur content	120 h, pH7, 30 °C, 147 rpm, 1/1 (O/W), resting cells

Analytical techniques

Cell growth was measured by total viable count (cells/mL) via serial dilutions (10^{-1}) on Luria Bertani/agar plates (Ashutosh Bahuguna et al. 2011). The growth rate was calculated using the exponential growth equation (Bailey and Ollis 1986):

$$ln\left(\frac{X_t}{X_o}\right) = k_g t$$

where X_o and X_t are the biomass concentration (i.e., total viable count cells/mL) at initial and final time (t, h), respectively, and k_g is the kinetic growth constant (h^{-1}).

The cultures pH was monitored at the prescribed time intervals with pH-meter (DIGMED DM-22, São Paulo, Brazil). Gas chromatography–mass spectrometry (GC/MS; Perkin Elmer Clarus 500 MS System, USA) applied to elucidate the DBT-BDS pathway (El-Gendy et al. 2014). High-performance liquid chromatographic analysis (HPLC; Waters 600 E equipped with a dual-wavelength UV detector model Waters 2487, Waters Corporation, MA, USA) was used to follow up the BDS of thiophenic compounds in model oil (Nassar et al. 2017). The total sulfur content in real oil feed was determined by Energy Dispersive X-Ray Fluorescence EDXRF (model Spectroscan S (SL), Spectron, Russia) according to the ASTM D4294 – 03. BDS was expressed as:

$$\mathrm{BDS}\% = \left(\frac{(\text{Intial sulfur concentration} - \text{Final sulfur concentration})}{\text{Intial sulfur concnetration}}\right) \times 100$$

The rate of sulfur removal was calculated applying the first-order kinetic model equation: ($S_t = S_o e^{-kt}$), and the half-life time ($t_{1/2}$) was calculated from: ($t_{1/2} = \frac{\ln(2)}{k}$), where S_o and S_t are the initial and sulfur concentration ate time t (h), respectively and k is rate constant h^{-1}.

Biosurfactant(s) production was detected in the cultures by measuring the surface tension of the medium using a Du-Noüy ring-type tensiometer (Krüss type K6 GmbH, Hamburg, Germany) (Ali et al. 2014). To assure the selective BDS without affecting the hydrocarbon skelton, calorific value was measured before and after the biodesulfurization process, according to the ASTM D240-19 using calorific value tester (Parr 6200, Parr Instrument Company, IL, USA) and the hydrocarbon skeleton was monitored according El-Gendy and Nassar (2015) by using gas chromatography with flame ionization detector (GC-FID; model Agilent 6890 plus, NY, USA), equipped with a capillary column HP-1 (100% methyl silicon siloxane, 30 m × 0.25 mm; Hewlett-Packard, USA).

Modelling approach and statistical analysis

SPSS version 13.0 (Informer Technologies, Inc., Los Angeles, CA, USA) was used for multiple comparisons applying Tukey's method to evaluate the significant difference between levels in each studied parameter. The mean difference is significant at the 0.05 level and 95% confidence interval.

Response surface methodology (RSM) based on the OFAT was applied to model mathematical correlations describing the response variable (BDS efficiency) as a function of each of the studied operational conditions (independent variables). The statistical software Design Expert 6.0.7 (State-Ease Inc., Minneapolis, MN, USA) was used for the elucidation of mathematical modelling, regression, and graphical analyses of the data obtained and statistical analysis of the predicted models to assess the analysis of variance (ANOVA). The validity of the fitted models was evaluated, and their statistical significance were controlled by F test. The predicted mathematical model equations' applicability and reliability were validated by a high correlation coefficient (R^2) and adjusted correlation coefficient (R^2_{adj}) and a low standard deviation and also a high agreement between the calculated and experimental values.

Results and discussion

Biodesulfurizing bacterium isolation and identification

A white, smooth, opaque, flat, Gram-positive, rod-shaped, spore-forming (Fig. 1), motile, halotolerant, mesophilic, neutrophilic, and facultative anaerobic bacterium was isolated from the collected oil-polluted sediment sample. The 16S rDNA identified that isolate as *Paenibacillus glucanolyticus* strain HN4 (NCBI Gene Bank Accession No. MT645230) with a percent similarity of 99.24%; based on its phylogenetic tree (Fig. 1). The GC/MS evidenced the ability of HN4 to desulfurize DBT without attacking its hydrocarbon skeleton, producing 2-hydroxybiphenyl (2-HBP) as a dead-end product through the 4S-pathway (Fig. 2). According to Su et al. (2018), such a non-destructive pathway occurs through two cytoplasmic monooxygenases (DszC and DszA) and a desulfinase (DszB) (Fig. 1), which are governed by an operon (*dsz*ABC) and free cell oxygen transport, and uptake is necessary because the 4S pathway is an oxidative pathway that requires 3 moles of O_2 per mole of DBT desulfurized (Bhanjadeo et al. 2018).

A thermophilic *Paenibacillus* sp. strain A11-2 has been reported as a selective efficient biodesulfurizing bacterium for DBT and its alkylated derivatives (Onaka et al. 2001). Wang et al. (2015) 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. (2019) from a polluted marine environment for bioremediation of oil hydrocarbons polluted soil. Nevertheless, as far as our knowledge, no BDS research studies have been reported using *Paenibacillus glucanolyticus*. Thus, strain HN4 is the first *P. glucanolyticus*

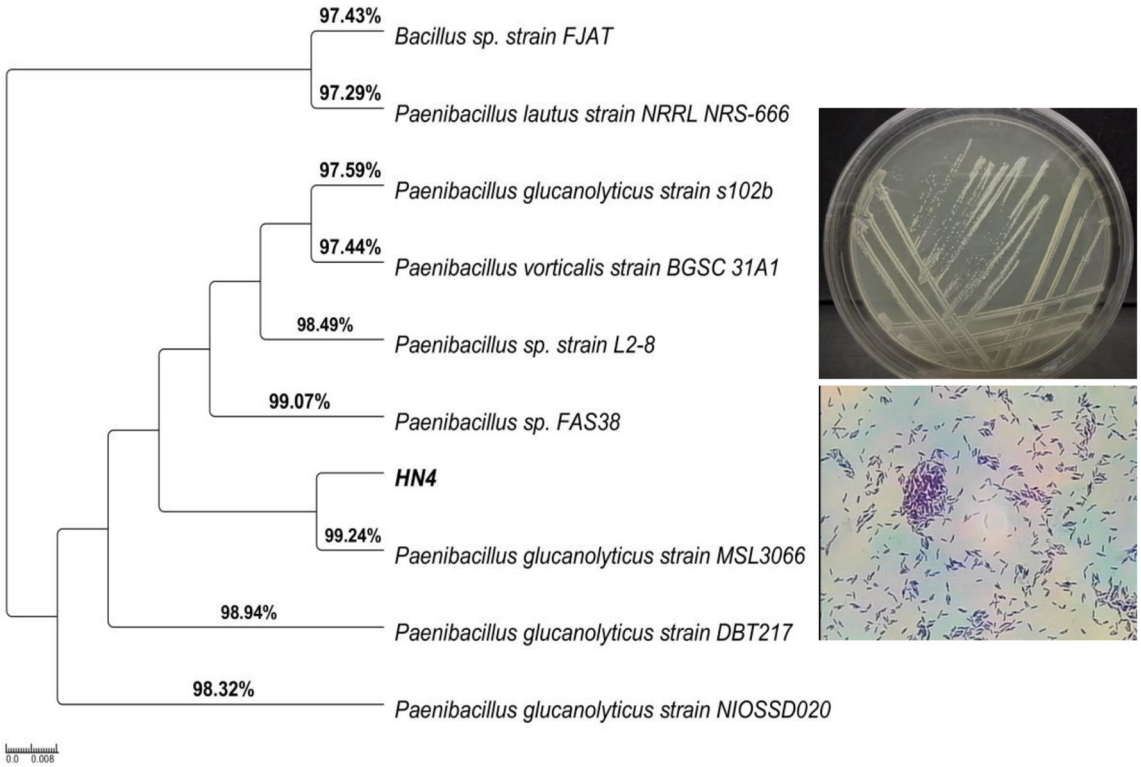


Fig. 1 Phylogenetic tree constructed by neighbour joining method of 16S rDNA gene for HN4 and closely related bacteria

reported for selective desulfurization of DBT via the 4S-pathway in a biphasic system without affecting its hydrocarbon skeleton.

Optimization of batch biphasic BDS process

It is essential to determine the abiotic loss of the targeted substrates in non-inoculated flasks to exactly conclude the bioactivity of the injected biocatalyst, and the recorded average abiotic loss for thiophinic compounds in all performed experiments in this study was negligible (< 1%). Thus, any observed loss exceeding this value in the inoculated flasks was attributed to the BDS-process. Culture pH is a vital factor in a bioprocess for maintaining the injected biocatalyst's maximum enzymatic activity (Barrios 2011). Advantageously, in this study, there was no significant change in pH during the BDS batch processes. A similar observation reported by Kim et al. (2004) and was attributed to the used BSM's adequate buffering capacity.

The growth kinetics with depletion of substrate and production of the end product are essential inputs for any biological reactor's design. The change of biodesulfurization efficiency with the growth profile is illustrated in Fig. 3. The biphasic BDS system (0.1 wt% DBT in n-hexadecane 1/4 O/w) using growing cells of *P. glucanolyticus* strain HN4 was found to follow first-order kinetic model equation (R^2 0.9397) with a rate constant of 0.0076 h^{-1} and $t_{1/2}$ 91.2 h. HN4 expressed an efficient growth rate of 0.0431 h^{-1}, which was corresponding to 16.08 h doubling time. The absence of lag phase evidenced the enriched enzymatic adaptability of HN4. There was a concomitant increase in microbial biomass with the depletion of DBT, recorded their maximum after 96 and 120 h, respectively and remained nearly stable at longer incubation period. This might be attributed to the accumulation of 2-HBP with time, which would inhibit the microbial growth and enzymatic system via the feedback inhibition phenomenon (Chen et al. 2019).

Nevertheless, there was a time span delay between the initial production of 2-HBP and DBT depletion of approximately 48 h. This might be attributed to the slow release of 2-HBP from the cells (Sharma et al. 2020), its accumulation onto the cell surface (Wang et al. 2015) and/or the adsorption of DBT onto the bacterial cell wall (Mohamed et al. 2015). However, the maximum production of 2-HBP (370 mg/L, i.e. 2.17 mM), which is around 53% of the transformed DBT, occurred at the late exponential phase (120 h) and remained nearly sustained thereafter. A similar observation has been reported by Nakashima and Tamur (2004) and Ismail et al. (2016), as due to the usual occurrence of intracellular accumulation of 2-HBP, approximately half of the desulfurized DBT are detected as a biotransformed 2-HBP.

Based on the results obtained by Tukey's method; the best operational time for BDS% was within the exponential phase (120 h), while the effect of longer operational time (> 120) was not significant (p = 0.953 and 0.162, between 120 and 144, and between 144 and 168 h, respectively). The recorded high BDS

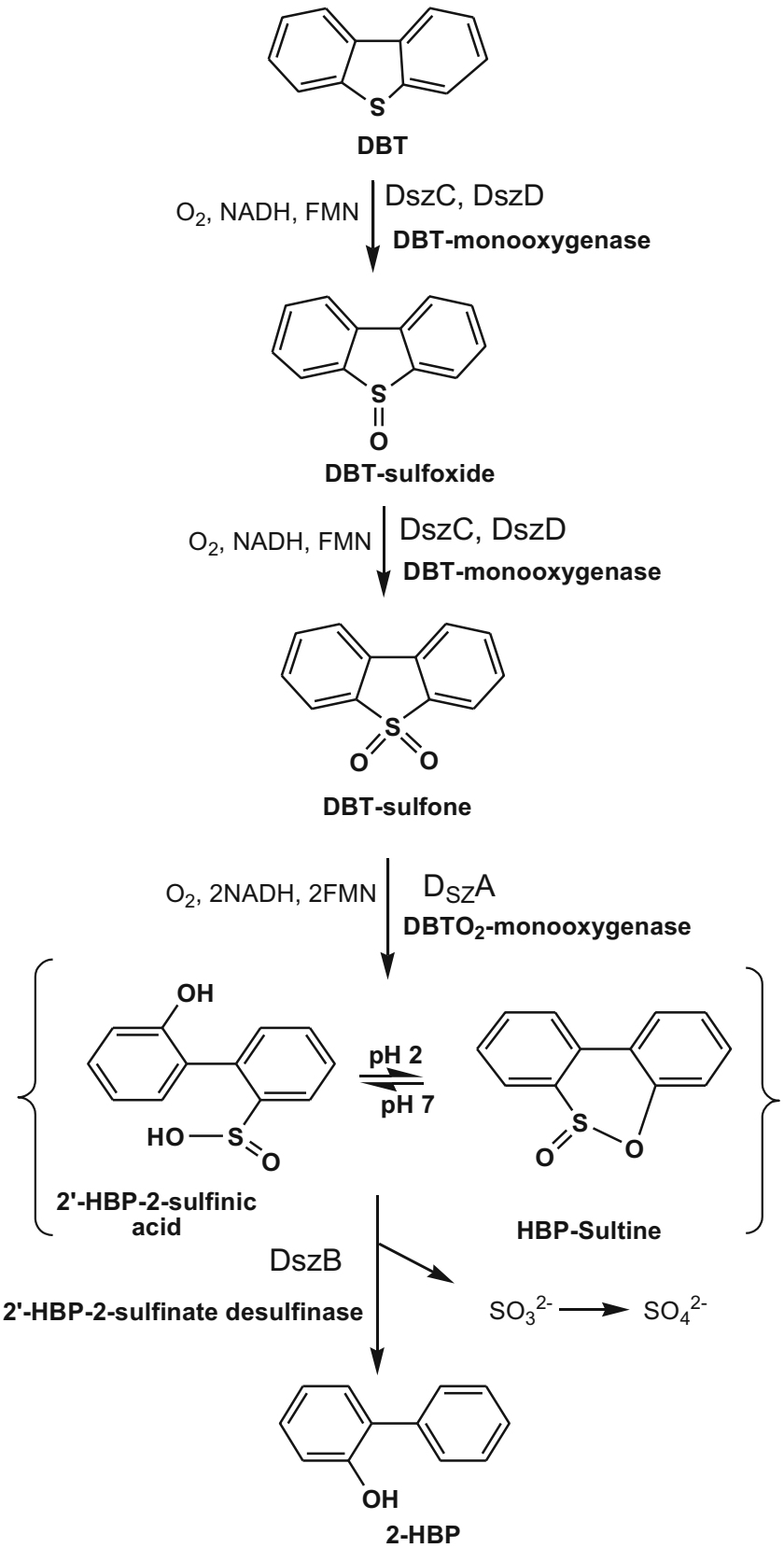


Fig. 2 Elucidated DBT-BDS pathway by *Paenibacillus glucanolyticus* strain HN4

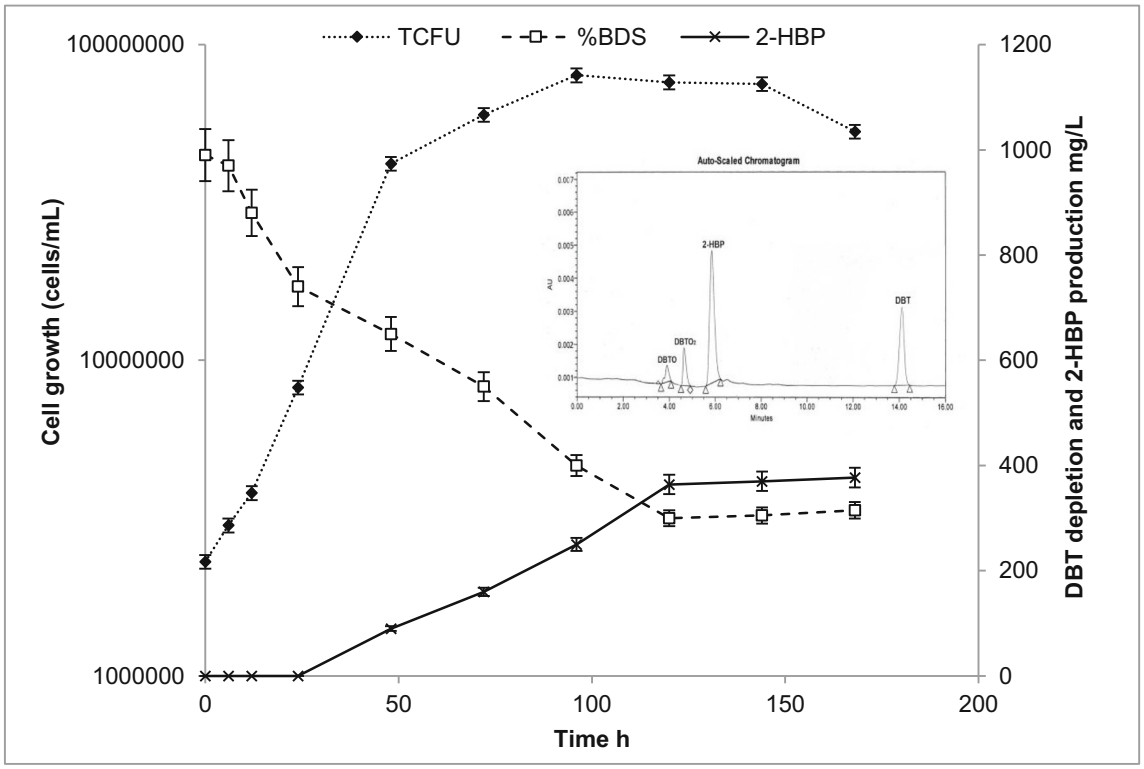


Fig. 3 The batch biphasic biodesulfurization time profile of *Paenibacillus glucanolyticus* strain HN4 pH7, 30 °C, 150 rpm, 1/4 (O/W), 0.1 wt% DBT (5.43 M), growing cells

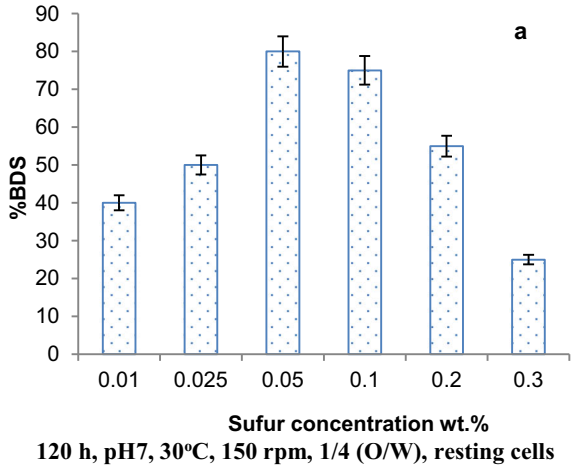
rate during the logarithmic growth phase has also been reported by Maass et al. (2015). Thus, the late exponential phase (120 h) was chosen in this study to harvest enriched resting cells for feeding the BDS reactors, as it was the time recording the maximum enzymatic activity, i.e. BDS effeceincy. Resting cells have been reported to offer higher desulfurization efficiency than the growing ones, as it overcomes the problem of cofactor regenerations and consequently enhances the three first oxygenation steps in the 4S-pathway (Ohshiro et al. 1996; Konishi et al. 1997; Luo et al. 2003; Alcon et al. 2005; Calzada et al. 2009). Moreover, the aqueous phase can also be reduced upon the usage of high densities of resting cells (Caro et al. 2007). No significant peaks appeared in the HPLC chromatogram other than the DBT and 2-HBP (Fig. 3), confirming the fast and immediate consumption of the three first oxygenation steps intermediates (DBTO, $DBTO_2$, and 2′-hydroxybiphenyl-2-sulfinic acid HPBSi). Consequently, 2-HBP as the dead-end product of the BDS reaction was the only measured product with the DBT-depletion and the microbial growth increment. Moreover, the data proved the efficient tolerance of HN4 to high concentrations of toxic 2-HBP, even in aqueous system, where, HN4 removed 60% of 5.43 M DBT with a sufficient concomitant growth recording 5×10^7 cells/ml and a production of 245 mg/L 2-HBP (i.e. 1.44 mM) after 120 h at 30 °C and pH 7. Derikvand et al. (2015) reported maximum production of 2-HBP (0.27 mM) from 0.41 mM DBT by growing cells of *Paenibacillus validus* Strain PD2 after 50 h of incubation at 31.23 °C and pH 6.92. It was noticed that the BDS efficiency in biphasic system is relatively higher than that of aqueous system. Similar observation was also reported by Derikvand et al. (2015), upon using DBT dissolved in n-tetradecane and attributed this to the reduction in toxic effect of DBT and its metabolites as they are dissolved in the organic phase.

HN4 was observed to adhere to the oil-water interface and produced a robust stable emulsion. That would be an indirect evidence for biosurfactants(s) production (Kaufman et al. 1998). That adds also to the advantageous of HN4 as according to Monticello and Kilbane (1994) formation of bioemulsions enhances the rate of BDS as it overcomes the mass transfer limitation. HN4 showed by GC/MS analysis to have the ability to produce 2′-HBP-2-sulfinic acid (HBPSi), which is reported to be a starting material for novel surfactants (Monticello 2000; Kawaguchi et al. 2012). From the practical point of view, to save time and energy, the optimum operating time was set 120 h for the forthcoming experiments.

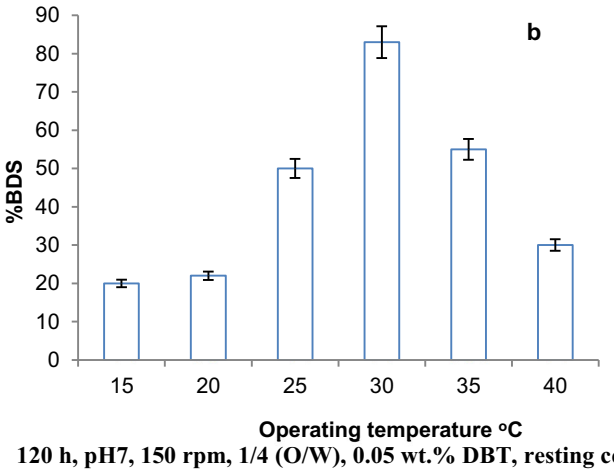
The analysis of variance (ANOVA) proved that there are statistically significant differences between all of the studied parameters ($p < 0.0001$). The effect of incubation time, operating pH, and mixing rate were found to have a highly statistically significant effect on BDS-efficiency ($p < 0.0001$). The statistical significant effect of the other studied parameters can be ranked in the following order; different S-concentration ($p = 0.0019$) > operational temperature ($p = 0.0134$) > oil/water phase ratio (p

= 0.032). That might indicate the tolerance and broad versatility of HN4 enzymatic system to desulfurize recalcitrant thiophenic compounds over a considerably wide range of operating temperatures, high concentrations of sulfur, and oil/water phase ratio, which consequently adds to the advantageous of HN4 and favorably recommends its application in oil refineries.

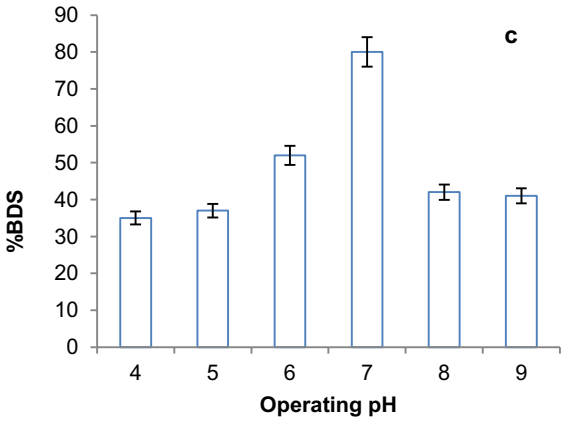
The optimum operating conditions were found to be 0.05 wt% DBT, pH7, 30 °C, where it recorded approximately 80% BDS efficiency (Fig. 4a–c). However, it sharply decreased at higher sulfur concentrations (≥ 0.2 wt%; Fig. 4a). For different S-concentration, there was a very high statistical significant differences between all the studied concentrations



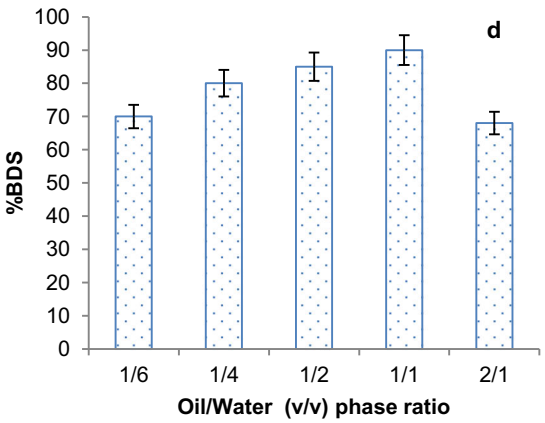
120 h, pH7, 30°C, 150 rpm, 1/4 (O/W), resting cells



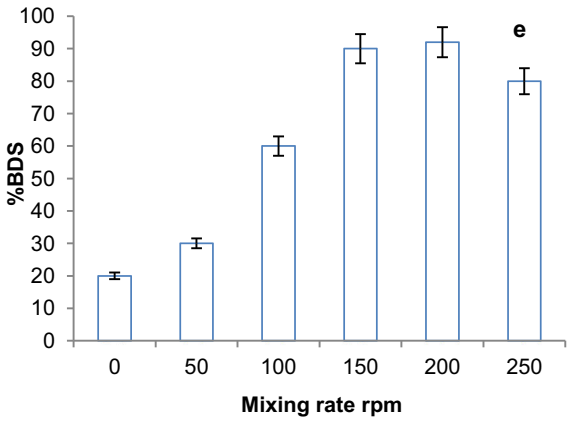
120 h, pH7, 150 rpm, 1/4 (O/W), 0.05 wt.% DBT, resting cells



120 h, 30°C, 150 rpm, 1/4 (O/W), 0.05 wt.% DBT, resting cells



120 h, pH7, 30°C, 150 rpm, 0.05 wt.% DBT, resting cells



120 h, pH7, 30°C, 1/1 (O/W), 0.05 wt.% DBT, resting cells

Fig. 4 Effect of sulfur concentration (**a**), operating temperature (**b**), operating pH (**c**), oil/water phase ratio (**d**), and mixing rate (**e**) on BDS efficiency

($p < 0.0001$) except that between 0.025 and 0.2 wt% and between 0.05 and 0.1 wt% ($p = 0.001$). The process temperature is reported to significantly impact the BDS rate-limiting first and third enzymes (DszC and DszB) of the 4S pathway (Furuya et al. 2001). For operational temperature (Fig. 4b) Tukey's method for statistical analysis showed that there was no significant difference between 15 and 20 °C ($p = 0.083$) means that the operational temperature under 20 °C can be neglected in future work. Nevertheless, there was a very highly statistical significant differences between 30 °C and all other operation temperatures ($p < 0.0001$), and there was also a statistical significant difference between 25 and 35 °C ($p = 0.001$). The changes in pH would affect the enzymes' 3-D shape and the electrical charge on the substrate, which would consequently retard or inhibit the substrate binding to enzymes' active sites. Thus, inhibit the enzymatic catalytic activities (Derikvand et al. 2015). Besides, the 4S-pathway enzymes are reported to be active at pH around neutrality (pH6-8) (Matsubara et al. 2001). The effect of the pH variation (Fig. 4c) was very highly significant ($p < 0.0001$) except the value between acidic pH 4 and 5 which was not significant ($p = 0.147$). Consequently, it is not necessary to perform the experiment with both values and can select either 4 or 5 only. Same occurred for alkaline pH 8 and 9 ($p = 0.434$). Thus pH higher than 8 and below 5 can be neglected in future work. Resting cells of *Paenibacillus validus* (strain PD2) that was previously isolated from oil-polluted soil for its ability to desulfurize DBT via 4S-pathway, and also producing 2-HBP as a dead-end product, revealed relatively similar optimum operating conditions to those of HN4; 7.86 mM DBT, pH 6.62, and 27.73°C at a 1/2 O/W biphasic BDS process (Derikvand et al. 2015).

The BDS efficiency increased with O/W phase ratio recording, approximately 90% at 1/1 O/W (Fig. 4d). This would prove the considerable tolerance of *P. glucanolyticus* strain HN4 to high concentrations of hydrocarbons and solvents. Nevertheless, the BDS efficiency decreased by approximately 22%; at a higher O/W phase ratio. Further, Tukey's results revealed that the difference between 1/6 and 2/1 was not statistical significant ($p = 0.092$). But there were statistical significant differences between 1/2 and 1/4 and 1/1 and 1/2 ($p = 0.002$). The recorded high BDS efficiency at high O/W would indicate that HN4 overcome the mass transfer obstacle of sulfur compounds from the oil phase to the aqueous phase at a relatively high O/W phase ratio. Besides, it might also indicate that HN4 has overcome the expected limitation of oxygen transfer at high O/W phase ratio as it is a facultative anaerobe (Abin-Fuentes et al. 2014).

Tukey's method for statistical analysis proved that the difference between all operational mixing rates was very significant, with $p < 0.0001$ except the difference between 150 and 200 ($p = 0.104$). Thus, from the economic point of view, as long as there is no significant difference in BDS efficiency within the mixing rate (150–200 rpm; Fig. 4e), 150 rpm would be considered the optimum. Nevertheless, at a higher mixing rate (> 200 rpm), the turbulence increase would be harmful to bacteria, causing cell rupture, and the mass-transfer limiting the BDS-process would be turned to reaction control (Nassar et al. 2017).

Modelling approach and validation

The predicted mathematical model equations that are best representing the effects of different studied physicochemical operational parameters on batch biphasic-BDS are illustrated in Table 2. The F test and p values evaluated the statistical significance of the predicted model equations for analysis of variance (ANOVA). They were found to be statistically significant at a 95% confidence level, with high F values and low probability p values (Table 2). The high values of the determination coefficients, R^2 (≥ 0.9571), and R^2_{adj} (≥ 0.9250) proved the models fitting reliability. This suggests that approximately 95.71% of the variance is attributed to the variables and indicated a high significance of the elucidated models. Thus, less than 0.0429 of the total variations cannot be explained by the predicted model correlations, which ensured the predicted models' reasonable adjustment to the obtained experimental data. Confirmation of the regression models' adequacy is also reflected by the excellent agreement between experimental and predicted response variable (i.e. predicted and actual BDS effeceincy), as shown in Fig. 5. The adequacy precision measures the signal to noise ratio. A ratio greater than 4 is desirable. As can be seen from Table 2, all the calculated adequacy precision ≥ 10.857; thus, all predicted model equations are reliable and can be used to navigate the design space. Determination of coefficient of variance is essential and measures the reproducibility of the model. It indicates the ratio between the standard error of estimate with the mean value of the observed response percentage. If the value of the coefficient of variance is less than 10%, then the model can be considered reasonably reproducible. The low values of the standard deviation and the coefficient of variance (Table 2) indicated the predicted models' high precision and reproducibility. For optimization, each operational condition's desired goal was chosen within the studied range, and the response was defined as the maximum to achieve the highest BDS-efficiency. Accordingly, the predicted maximum BDS efficiency was found to be 92% at the calculated optimum operating conditions; 120 h, 33.5 °C, 0.14 wt% S-concentration, pH7, 1/1 O/W, and 147 rpm, with a desirability function value of 1.000. At the same time, the BD efficiency of the performed experimental run at such predicted optimum conditions recorded 94%. As far as our knowledge, *Paenibacillus glucanolyticus* strain HN4 is the first to be isolated for selective and effective BDS of different

Table 2 The predicted mathematical correlations with their corresponding goodness of fit parameters

Parameter	Model equation	Model validation
Incubation period	$BDS\ \% = 55.03 + 33.98A - 19.26A^2$	F value 224.79, $p < 0.0001$ Standard deviation 3.49 R^2 0.9868, R^2_{adj} 0.9824 Coefficient of variance 8.13 Adequacy precision 33.764
DBT concentrations	$BDS\ \% = 76.03 - 61.13B - 44.27B^2 + 54.39B^3$	F value 70.68, $p = 0.0006$ Standard Deviation 3.72 R^2 0.9815, R^2_{adj} 0.9676 Coefficient of variance 7.63 Adequacy precision 19.552
Operating temperature	$BDS\ \% = 7.83 + 3.36C - 2.95C^2 - 2.87C^3$	F value 29.77 $p = 0.0034$ Standard deviation 0.14 R^2 0.9571, R^2_{adj} 0.9250 Coefficient of variance 3.99 Adequacy precision 12.833
Operating pH	$BDS\ \% = 67.27 + 65.88D - 29.15D^2 - 62.93D^3$	F value 210.96, $p < 0.0001$ Standard deviation 1.58 R^2 0.9937, R^2_{adj} 0.9890 Coefficient of variance 3.40 Adequacy precision 10.857
Oil/water (O/W) phase ratio	$BDS\ \% = 86.88 + 13.67E - 17.57E^2 - 14.67E^3$	F value 36.25, $p = 0.0023$ Standard deviation 1.26 R^2 0.9645, R^2_{adj} 0.9379 Coefficient of variance 2.91 Adequacy precision 12.105
Mixing rate	$BDS\ \% = 76.96 + 90.42F - 27.10F^2 - 60.35F^3$	F value 950.67, $p < 0.0001$ Standard deviation 1.91 R^2 0.9975, R^2_{adj} 0.9956 Coefficient of variance 3.46 Adequacy precision 54.587

thiophenic compounds at elevated oil/phase ratio, and under mesophilic conditions.

Biodesulfurization of model oil with a mixture of thiophenic compounds

It is evident from Fig. 6 that HN4 has a wide versatility to desulfurize different thiophenic compounds. That suggests that HN4 may have a novel enzymatic system for BDS of heterocyclic sulfur-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 (Kobayashi et al. 2001; Zhang et al. 2013). A similar observation was reported by Konishi et al. (1997) using thermophilic *Paenibacillus* strains in a biphasic system of DBTs dissolved in n-tetradecane. Konishi et al. (2000) also reported similar results to our findings, where *Paenibacillus* sp. strain A11-2 selectively desulfurize BT more efficiently than DBT. To our knowledge, few biodesulfurizing native strains can desulfurize both BTs and DBTs, for example *Rhodococcus erythropolis* KA2-5-1 (Kobayashi et al. 2000), *Rhodococcus* sp. KT462 (Tanaka et al. 2002), *Sphingomonas subarctica* T7b (Gunam et al. 2006), *Mycobacterium goodii* X7B (Li et al. 2006), *Bacillus subtilis* WU-S2B (Ohshiro et al. 2009), *Brevibacillus invocatus* C19 (Nassar et al. 2013), *Gordonia* sp. HS126-4 N (Akhtar et al. 2018), and *Gordonia* sp. SC-10 (Chen et al. 2019). It is also known that high molecular weight alkylated DBTs, especially those alkylated at the 4- and/or 6-position, are challenging to be biodesulfurized by most of the biodesulfurizing microorganisms (Chen et al. 2018). Thus, *Paenibacillus glucanolyticus* strain HN4 with its efficient BDS capacities towards such alkylated-DBTs is considered a real and efficient added value to the BDS field where thiophenic compounds can represent up to 70% of the total organic S-compounds in crude oil and its fractions.

Results of Tukey's method for statistical analysis revealed that there was no statistically significant difference between the BDS efficiencies of Th, BT, and DBT ($p = 0.155$). That attested the broad versatility of the enzymatic system of HN4. Nevertheless, there was a very highly significant difference between the BDS efficiencies of those thiophenic compounds and 4,6-DMDBT ($p < 0.0001$). That ascertained the negative

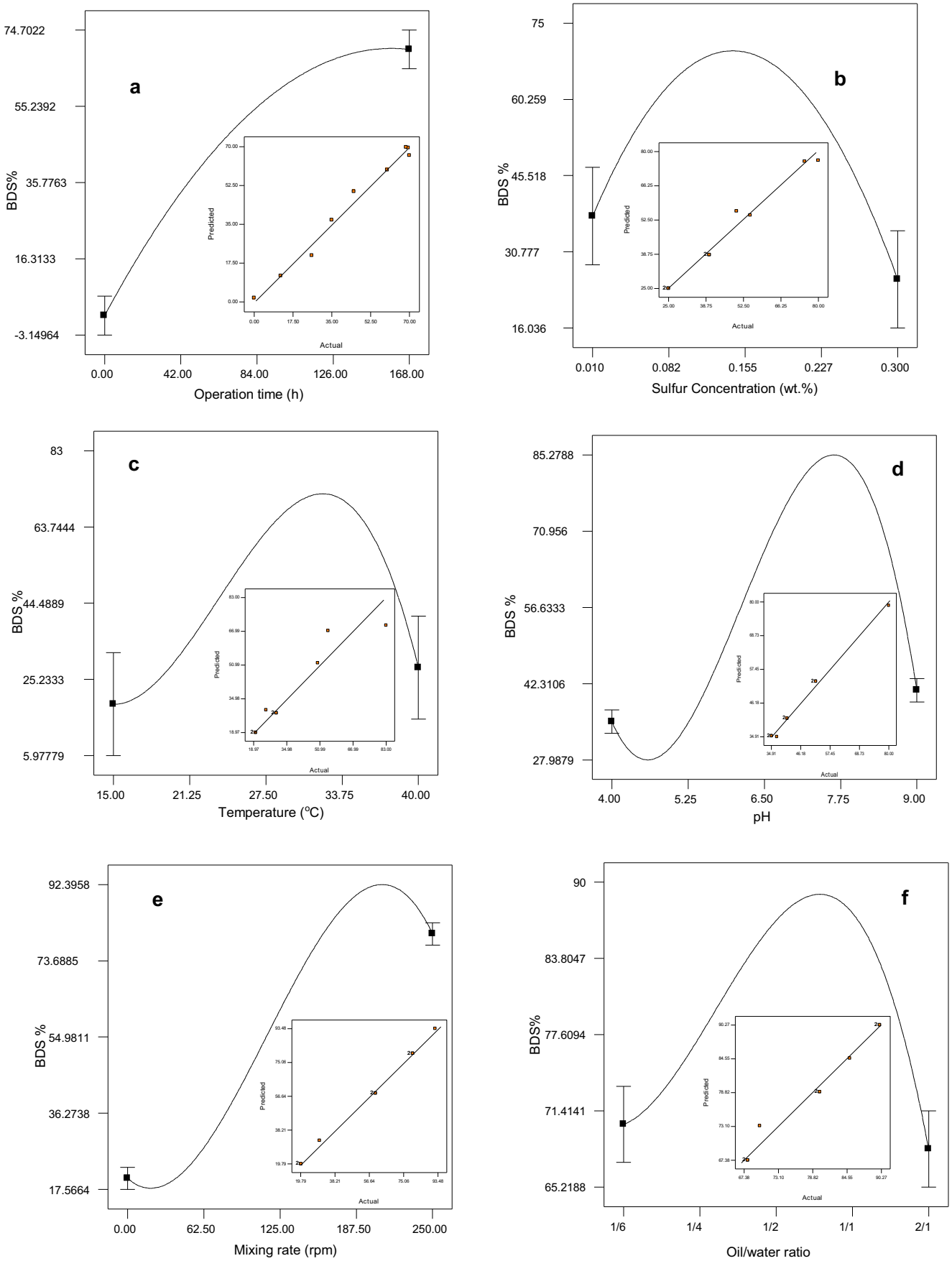


Fig. 5 Predicted model plots with experimental versus calculated BDS efficiency

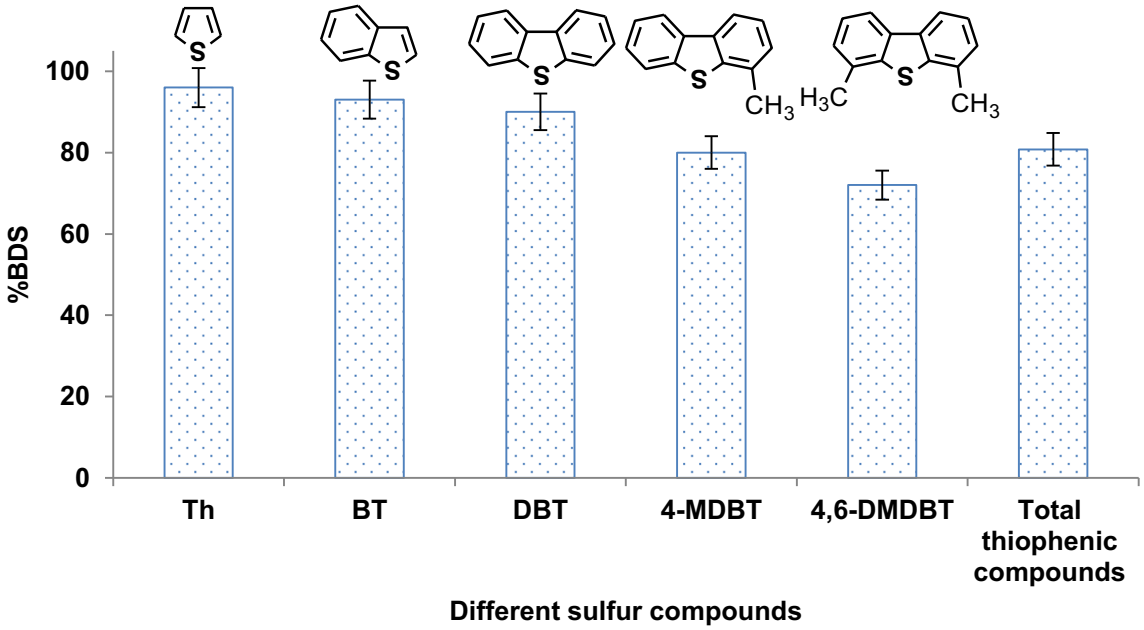


Fig. 6 Biodesulfurization of thiophenic model oil *by Paenibacillus glucanolyticus* strain HN4

impact of the two methyl groups' steric hindrance effect at 4 and 6 positions on the enzymatic attack of HN4 to S–C bond. However, there was no significant difference between the BDS efficiencies of alkylated DBTs (4-MDBT and 4,6-DMDBT) ($p = 0.109$). That evidenced the recalcitrant hydrocarbon tolerance of HN4 and its enzymatic adaption to desulfurize such recalcitrant compounds.

The surface tension of the culture decreased by 45%, which might substantiate the production of biosurfactants(s), which would enhance the bioavailability of the S-compounds and mass transfer, consequently enhance the BDS rate. A similar observation was reported by Agarwal and Sharma (2010) and Amin et al. (2013) and explained the increase of BDS rate by the amended solubility of hydrophobic substrates in water via the development of reversed micelles which enriched the surface area of the 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. Moreover, the observed production of biosurfactants(s) would explain the tolerance of HN4 for high DBT and hydrocarbon concentrations, as biosurfactants might have limited their toxicity and inhibitory effects.

Biodesulfurization of real petro-diesel oil feed

Upon the application of HN4 in a biphasic (1/1 v/v) batch BDS of a real petro-diesel oil, the sulfur content decreased from 0.2 to 0.04 wt% within a 120 h, without any adverse effect on the calorific value of the desulfurized oil, recording 45.69 MJ/kg. The gas chromatographic analysis using GC/FID revealed a negligible degradation in the light n- and iso-alkanes ($\leq C_{17}$) recording 14 and 7%, respectively without any remarkable effect on the unresolved complex mixture (UCM) components or the biomarkers pristane and phytane. Consequently, such slight degradation in oil feed alkanes enhanced the diesel oil BDS, as according to Setti et al. (1995), substrate uptake presumably occurs first by the adsorption of the microorganism to the oil-phase, followed by diffusion or active transport at the point of contact (Fig. 7). Consequently, microorganisms adhere to the n-alkanes, usually those below C_{16} which form a film around the aromatic sulfur compound, and as much as the microorganisms easily attack this film, the bioavailability of sulfur compounds increases. This is also reported to be enhanced by the production of biosurfactants, which increase the bioavailability of the thiophenic compounds by increasing the contact between oil and aqueous phase, increasing the cell hydrophobicity, and decreasing the mass transfer limitation, with a consequent increase in the diffusion of DBT into the aqueous phase and avoided accumulation of 2-HBP, thus improving the BDS yield (Li and Jiang 2013). Besides, the presence of carbon co-substrate in the BDS media like glycerol promotes the oxidoreductase activity and enhances the production of the enzyme co-factors; NADH and $FMNH_2$ (Bordoloi et al. 2014).

Thermophilic *Paenibacillus* strains were previously reported as an efficient BDS on hydrodesulfurized light gas oil at 50 °C, which recorded a decrease in sulfur content from 800 to 720 ppm at 20% O/W reactor (Konishi et al. 1997). The recorded efficient BDS activity by HN4 with its high hydrocarbon tolerance and wide versatility demonstrated 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

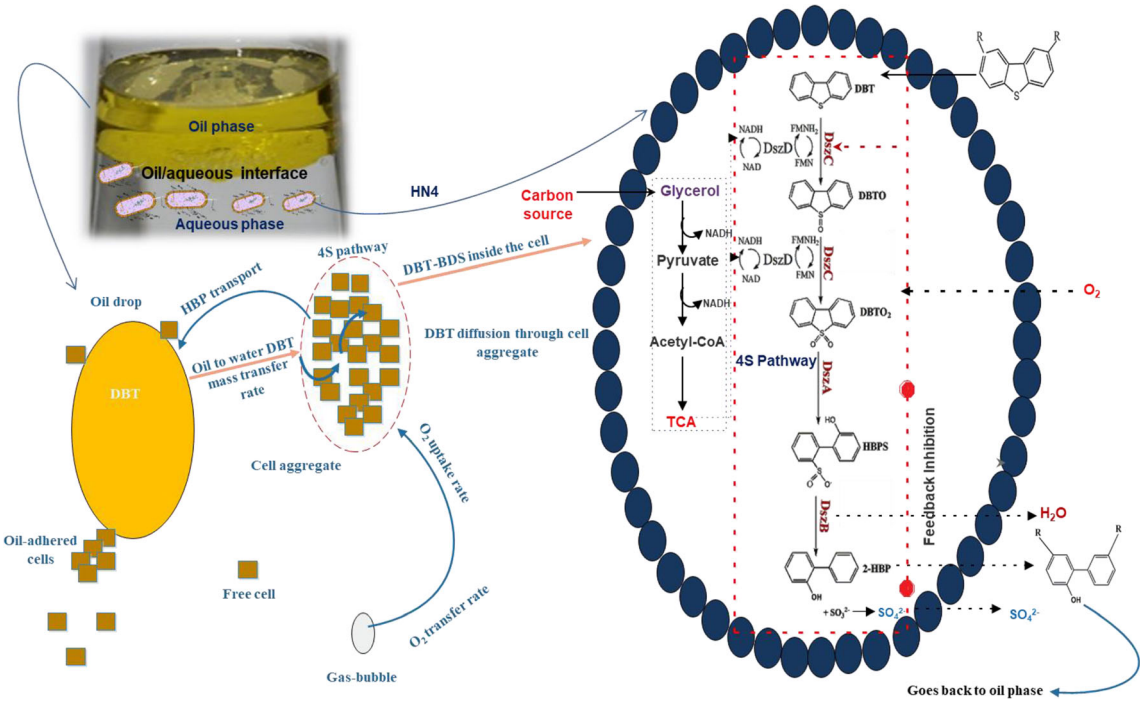


Fig. 7 Presumptive mechanism for petro-diesel BDS by *Paenibacillus glucanolyticus* strain HN4 (R may be an alkyl or H)

micro-flora. Consequently, the presence and abundance of microbial strains with high tolerance to recalcitrant oil components in such oil-contaminated habitats would support the isolation of microbial biocatalyst characterized by an activated and enriched enzymatic system tolerating 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. 3), indicating the good endurance and adaptation of *Paenibacillus glucanolyticus* strain HN4, considering that DBT was supplemented to the enriching medium used for HN4 isolation, which enhances the selectivity of the isolate.

Conclusion

The ability of the newly isolated *P. glucanolyticus* strain HN4 to release sulfur from DBT as a model of thiophenic compounds in petro-diesel, without attacking its hydrocarbon skeleton and its efficient capability to remove approximately 80% of the sulfur content of real oil feed, recommend its application for BDS of oil and its fractions without affecting its calorific value. Besides, its high hydrocarbon tolerance and biosurfactant(s) production increase its chance for industrial application, which adds to the advantage of HN4 as it will decrease the consumption of water during the BDS process and desulfurize higher amounts of oil feed per each batch. Thus, increase the feasibility of the BDS process. However, further work is undertaken now to investigate the feasibility of the repeated continuous and/or batch desulfurization of the same oil feed with immobilized and free cells, to reach to ultra-low diesel oil and to overcome the problem of free cell separation in BDS emulsions, as it consumes high energy, during the ultra-speed centrifugation. The broad versatility of HN4 to desulfurize different heterocyclic S-compounds, especially the recalcitrant alkylated DBTs, also adds to its favourability for industrial application as complementary to the hydrodesulfurization process. Nevertheless, further work is required on its enzymatic and genetic system involved in BDS of DBTs and BTs.

Authors' contributions HNN conducted the isolation of the bacteria and its identification, all the microbiological and biodesulfurization experiments, all the analysis to follow up the biodesulfurization efficiency, and the kinetic analysis and its validation. SSA performed the statistical analysis. NShE conceived and designed the research, interpreted, analysed, and discussed the obtained data and wrote the manuscript, and all authors commented on previous versions of the manuscript. Finally, all authors read and approved the manuscript.

Data availability All data generated or analysed during this study are included in this published article.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate Not applicable

Consent for publication Not applicable

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