REDUCTION OF SULFUR LEVELS IN KEROSENE BY *Pseudomonas sp* STRAIN IN AN AIRLIFT REACTOR

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***Abstract* — Combustion of organic sulfur from fossil fuels can produce acid rain that deteriorates the environment and infrastructure. Sulfur removal by microorganism has appeared as an alternative for this challenge. In this work, biodesulfurization of 50:50 water-kerosene emulsions were carried out at 100 mL scale and in a 0.01 m^3 airlift reactor with resting cells of the reference strain ATCC 39327 and *Pseudomonas* native strains Nº 02, 05 and 06. The reactor conditions were 30°C, pH 8.0 and 0.34 m^3 h^{-1} air flow. After 7 culture days, the mean sulfur removal for the strains Nº 06 and ATCC 39327 was 64 and 53%, respectively, with a mean calorific power loss of 4.5% for both strains. The use of the native strain Nº 06 and the designed airlift reactor is shown as an alternative for biodesulfurization process and constitute a first step for its scale-up to pilot plant.**

***Keywords* — Biodesulfurization, Airlift Reactor, Kerosene, *Pseudomonas*.**

I. INTRODUCTION

The main problem in fossil fuel combustion is sulfur and nitrogen conversion to oxides. These oxides make part of acid rain which deteriorates the environment and infrastructures (Monticello and Finnerty, 1985; Izumi *et al*., 1994; Oshiro *et al*., 1995; Sagardia *et al*., 1975). Furthermore, SO_2 emission is a precursor of sulfated aerosols that are considerated as one of the main solid particulate agents that affect the human health (Sagardia *et al*., 1975).

Worldwide sulfur levels in fuels are between 15 ppm in North America and 5000 ppm in some countries of Africa and Middle East (United Nations Environment Programme, 2007a). In Latin-America, Brazil, Bolivia, Chile, and Argentina have levels between 500 and 2000 ppm, while Venezuela, Ecuador, Peru, Paraguay, Uruguay and Centro-America have sulfur levels up to 5000 ppm in diesel. Mexico is the only Latin country with sulfur levels less than 500 ppm (Felix, 2007; United Nations Environment Programme 2007b). Since 1995, in Colombia some decrees have been created in order to control the sulfur level in fuels. They established for the year 2008 a level up to 300 and 500 ppm in gasoline and diesel, respectively. However, actual sulfur levels are 1200 ppm in Bogotá and 4500 ppm in other places (United Nations Environment Programme 2007b).

The microbiological degradation of sulfur compounds in fossil fuels, biodesulfurization (BDS), has arisen as an alternative instead of the catalytic process of hydrodesulfurization (HDS) which leaves sulfur remnants between 300 and 500 ppm (McFarland *et al*., 1998). In contrast, some microorganisms with sulfur removal activity show advantages like high sulfur specificity without fuel calorific power lost. BDS processes can be carried out under moderated conditions and allow to have lower sulfur remnant levels than HDS. However, these microorganisms have a high sensibility to organic solvents (Monticello, 2000). At present there are about fifty approved patents for desulfurization processes on derived fossil fuels from petroleum and coal which use microorganisms, enzymes and vectors that keep genes of desulfurization metabolic pathways (Biodesulfurization in United States Patent and Trademark, 2007). It is estimated that the BDS process could reduced CO_2 emissions and up to 80% less energy consumed per barrel compared to HDS (Le Borgne and Quintero, 2003).

In addition to elemental sulfur, sulfate, sulfite and thiosulfate, sulfur can be present in more than 200 organic compounds like sulfides, thiols, thiophenes, mercaptans, diphenilsulfides, benzothiophenes and dibenzothiophenes (Monticello and Finnerty, 1985; Fedorak and Westlake, 1983; Fedorak and Grbic-Galic, 1991; Lee *et al*., 1995). Dibenzothiophene (DBT) and its derivates have been the most studied compounds due their resistant to HDS process (Monticello, 2000). Three metabolic pathways for the desulfurization of DBT have been reported: (a) 4S pathway where the sulfur is selectively removed of the molecules without a significant lost in the fuel calorific power, (b) Kodoma's pathway where hydrophilic products are obtained with a lost in the fuel calorific power, and (c) a completely oxidative pathway where CO_2, $SO_3^=$ and H_2O are produced. (Oshiro *et al*., 1995; Lee *et al*., 1995; Armstron*set al*., 1995; Omori *et al*., 1992; Konishi *et al*., 1986; Kodama *et al*., 1973; Kodama *et al*., 1970). The enzymes and genes involved in 4S pathway have been all elucidated and constitute the Dsz desulfurization system that involves four enzymatic steps. Cofactors

participation (NADH and $FMNH_2$) and the complexity of the metabolic pathway force to work with whole cells (Le Borgne and Quintero, 2003).

The sulfur removal from fuels and organic solvents spiked with DBT or DBT-derived *eijerinkia* (Laborde and Gibson, 1977), *Rhodococcus* (Monticello and Finnerty, 1985; Fedorak and Westlake, 1983; Laborde and Gibson, 1977) *Desulfovibrio* (Armstrons *et al*., 1995), *Rhizobiummeliloti* (Frassinetti *et al*., 1998) and *Cunninghamella elegans* (Crawford and Gupta, 1990). *Pseudomonas* strains have shown BDS activity and resistance to organic solvents like n-decane, toluene, dimethylphtalate, etc., which represent an important characteristic for bioremedation processes. (Cruden *et al*., 1992; Fedorak and Grbic-Galic, 1991; Sagardia *et al*., 1975).

The choice of the process design for BDS depends on the economics of the production and recovery of the desired product. Recycling and operational stability of the biocatalysts are also important. Stirred tanks have been the most used reactors in BDS and there is just a few reports using airlift reactors, emulsion phase contactors and fluidized bed reactors (Le Borgne and Quintero, 2003; Monticello, 2000; McFarland *et al*., 1998). New processes include the use of multiple-staged airlift reactors to overcome poor reaction kinetics at low sulfur concentrations and reduce mixing costs (Monticello, 2000). Nevertheless, scale-up this process is a problem and there are not published reports that describe its engineering or operation to estimate it economically. The estimated capital costs for a BDS process is about two-thirds of a HDS process, whereas the operation cost is reduced by a 15% (Le Borgne and Quintero, 2003).

Previously, we reported the sulfur remove ability over n-hexane spiked with DBT of ATCC 39327 and 23 native strains of *Pseudomonas sp.* ATCC 39327 and native strains Nº 02, 05 and 06 used the 4S enzymatic pathway to remove sulfur in a 9.4, 6.0, 7.6 and 6.6%, respectively. Also, was proved that the proportion of 50:50 water:n-hexane and surfactant presence (ethanolamine oleate) had effect over the *Pseudomonas sp.* sulfur removal activity (Alméciga-Díaz *et al.*, 2005; Sánchez *et al.*, 2004).

In this work, the sulfur removal ability of ATCC 39327 and *Pseudomonas sp.* strains Nº 02, 05 and 06 over kerosene was initialed evaluated at 100 mL scale. Strain Nº 06 showed the highest sulfur removal levels and it was selected for BDS in an airlift reactor together with the reference strain. This system is shown as an alternative for biodesulfurization process and constitutes a first step for its scale-up to pilot plant**.**

II. METHODS

A. Microorganisms.

Pseudomonas sp. native strains and ATCC 39327 were stored in 10% free-fat milk and 3% meso-inositol at -20±1°C. Native strains were obtained from Corporación Autonoma Regional (CAR) wastewater ponds located in Cundinamarca, Colombia compounds have been reported for some strains of *Pseudomonas* (Huertas *et al*., 1998; McFarland *et al*., 1998; Izumi *et al*., 1994; Laborde and Gibson, 1977), *Corynebacterium* (Omori *et al*., 1992), *Arthrobacter* (Konishi *et al*., 1986), *B*
(Salazar, 1996). All microorganisms were biochemical characterized as *Pseudomonas sp.* (Sánchez *et al*., 2004).

B. Reactor Description.

Biodesulfurization assays were made in a concentric draft-tube airlift reactor (Fig. 1). Reactor vessel was 0.15 m in diameter and its overall height was 0.60 m. The draft-tube, 0.10 m in internal diameter and 0.30 m tall, was located 0.10 m above the bottom of the tank. The vessel was sparged in the concentric zone through a 0.005 m diameter sparger. The riser and downcomer area ratio was 0.80. The working volume and the overall volume of the reactor was 0.01 y 0.008 m^3, respectively. A dissolved oxygen electrode (HI8043 Dissolved Oxygen Meter, Hanna Instruments) and a pH-meter (HI98170 pH-meter with temperature probe, Hanna Instruments) were placed in the downcomer zone at 0.15 and 0.25 m above the base of the tank, respectively.

Air from ~700KPa mains was supplied to the reactor through a filter, pressure regulator, floor control valve and rotameter.

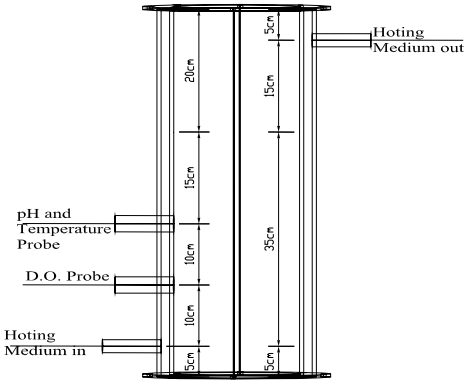

Figure 1. Airlift Reactor diagram.

C. Hydrodynamic Characterization

Mixing time and liquid circulation velocity was carried out with water, while the overall gas-liquid volumetric mass transfer coefficient (k_La) was carried out with water, kerosene and emulsion (50:50 kerosene:water and surfactant). These parameters were evaluated for three different air flows (0.142, 0.283 and 0.425 $m^3\ s^{-1}$) and measures were made by triplicate. Data were analyzed with Statgraphics Plus v. 5.0.

The mixing time and liquid velocity was determined by the acid tracer technique (Shariati *et al.,* 2007; Sánchez Mirón *et al.,* 2004; Chisti, 1989). The mixing time was defined like the time needed to reach 95% of the final tracer concentration at steady-state from the instance of tracer input. Once the reactor was filled with water, HCl (35 % $^{w}/_{v}$) was added until pH 2 and later air was bubbled by 20

minutes (0.34 m^3 h^{-1}) to remove any carbonate; later with NaOH (6 M) pH was established in 5.50±0.05. The acid tracer (1 mL of HCl 35% $^{w}/_{V}$) was added in the bioreactor through a port in the downcomer section. Dimensionless acid concentration [H+] was estimated with Eq. 1. While, mean liquid circulation velocity was calculated by Eq. 2.

$$C_T = \left[H^+\right] = \frac{\left[H^+\right]_{\text{at instant } t} - \left[H^+\right]_{\text{initial}}}{\left[H^+\right]_{\text{final}} - \left[H^+\right]_{\text{initial}}} \quad (1)$$

$$\overline{U}_{Lc} = \frac{x_c}{t_c} \quad (2)$$

where x_c is the circulation path length and t_c is the average time for one complete circulation.

The k_La was measured with the dynamic gassing-in method (Shariati *et al.*, 2007; Chisti and Jáuregui-Haza, 2002; Chisti, 1989). For each test the fluid was purged by bubbling nitrogen until a dissolved oxygen concentration lower than 5% of air saturation. Later the nitrogen flow was suspended, the exit of its bubbles was allowed and the air flow was established to the required condition. The increase in dissolved oxygen concentration was followed in the time until the fluid became nearly saturated with oxygen (>90%). The k_La was calculated as the slope of the linear equation:

$$-\ln(1-E) = k_L a\ (t - t_0) \quad (3)$$

In Eq. 3, E is the fractional approach to equilibrium (Chisti, 1989) and can be estimated Eq. 4.

$$E = \frac{C - C_0}{C^* - C} \quad (4)$$

where C^* is the saturation concentration of dissolved oxygen, C_0 the initial concentration of dissolved oxygen at time t_0 when a hydrodynamic steady-state has been reestablished (≤ 60 s) upon the beginning of aeration and C the dissolved oxygen concentration at any time *t*.

D. Pre-inoculum.

Microorganisms were initialed cultured in 0.3% CASO broth (Oxoid) at 30±1°C for 24 hours and then isolated in CVN agar (5% violet crystal solution, 2 mL L^{-1}; CASO, 3 g L^{-1}; agar-agar 15 g L^{-1}; 5% nitrofurantoine in dimethylformamide, 7mL L^{-1}) at 30±1°C for 24 hours. Finally a colony was inoculated in 200 mL of Luria-Bertani both (LB broth; Triptone 10 g L^{-1}; yeast extract 5 g L^{-1}; NaCl, 0.5 g L^{-1}; and glycerin, 1 mL L^{-1}) and incubated at 30±1°C and 200 rpm up to an OD_{600} 25±1%, equivalent to a resting cell population of 10^8 CFU mL^{-1} (Castro, 2000).

E. Fermentation Systems

Fermentations in 100 mL scale were carried out for each native and the reference strain by triplicate in 500 mL shake flasks. Culture medium was 50:50 aqueous: kerosene with 500 ppm of ethanolamine oleate (sur-factant). One milliliter of pre-inoculum (10^8 CFU mL^{-1}) was inoculated in the aqueous phase conformed by a free-sulfur minimum salt medium (FSMSM), (Frassinetti *et al.*, 1998). Shake flasks were incubated for seven days at 30±1°C and 200 rpm (Castro, 2000).

Fermentations in the airlift reactor were carried out in a 50:50 aqueous:kerosene ratio in presence of 3500 ppm of ethanolamine oleate. A 200 mL pre-inoculum of ATCC 39327 or strain Nº 6 was inoculated in 7.8 L of culture medium (FSMSM:kerosene). Fermentations were incubated for 7 days at 30°C and were carried out twice. A 50 mL sample was taken each 24 h for assays, and pH and dissolved oxygen were measured in the reactor. Air flow was 0.34 m^3 h^{-1}.

All the innoculus were carried out with resting cells (Le Borgne and Quintero, 2003).

F. Assays

Phases were separated by centrifugation at 3000 g for 30 minutes. Viability and sulfate quantification, by turbidimetry assay with barium chloride at 420 nm, were carried out in the aqueous phase. Qualitative analysis of kerosene was carried out by HPLC (Waters 600) in serial acetonitrile extractions of kerosene phase. Chromato-graphic system used a NovaPak C-18 column (30 cm x 3.9 mm x 5 μm) and a mobile phase with lineal elution gradient of water-acetonitrile (47:53) at a flow rate of 1 mL/min. Peaks were registered at 254 nm and scattered through 210 and 400 by a diodes array detector (DAD - Waters 996) coupled to the HPLC. Spectrum of each peak was compared to the standard solution spectrums of DBT, DBT-sulfone and 2-hydroxybiphenyl (Crawford and Gupta, 1990, Alméciga-Díaz *et al.*, 2005).

Kerosene calorific power was determinated in an adiabatic calorimetric pump (ICONTEC, 2003). Total sulfur was carried out by barium chloride in the condensed residue of the adiabatic calorimetric pump. Data were analyzed statistically using the software Statgraphics plus v. 5.0.

G. Viability

From the aqueous phase 100 μL were diluted with 900 μL of sterile water, serial dilutions were carried out up to 10^{-8} dilution and CFU count was measured after 12 hours incubation in CVN agar at 37ºC.

III. RESULTS AND DISCUSSION

A. Sulfur Removal from Kerosene in 100 mL scale

Initial kerosene calorific power and sulfur content was 49.7 KJ g^{-1} and 319 ppm, respectively. ATCC 39327 and strains Nº 02, 05 and 06 had an average sulfur removal of 19.9, 35.9, 34.0 and 62.6% and an average calorific power loss of 0.45, 5.55, 3.64 and 4.52%, respectively (Table 1). Kerosene chromatograms showed that neither DBT nor any of its metabolites were detected before and after culture, but after 7-culture days was noticed a decrease in the area of some non-identified peaks between 7.5 and 10.5 min.

For all strains, microorganism viability during the culture was around 10^8 CFU mL^{-1}. This result is likewise with previous reports using the same strains in n-hexane spiked with DBT (Alméciga-Díaz *et al.*,

2005; Sánchez *et al.*, 2005). Since the microorganisms kept their viability along the culture, they could become in a sulfur removal alternative for desulfurization studies in two-phase fermentation systems with high molecular weight hydrocarbons. Although, viability and sulfur removal results over kerosene have not been reported, similar results were reported for a *Psudomonas* strain in p-xylene 50% (Cruden *et al.,* 1992). However, *P. putida* and *P. mendocina* showed high sensitive to n-heptane concentrations greater than 10% (Huertas *et al.,* 1998).

Strains 02 and 06 produced the greatest calorific power lost, 5.5 and 4.5, respectively (Table 1). This could be associated with the use of fuel like a carbon source or the generation of free-sulfur hydrophilic compounds (Monticello, 2000). Although, there are no reports that allows saying whether this is an important calorific power lost or not, these values are lower that obtained in DBT chemical oxidation followed by solvent separation of the corresponding sulfones that led to a 10% decrease in the fuel energetic value (Le Borgne and Quintero, 2003).

B. Reactor Characterization.

For different air flows the designed reactor showed a circulation liquid velocity range between 0.060 and 0.095 m s^{-1} with a maximum standard deviation of 8%. The mixing time range was between 38 and 27 s with a maximum standard deviation of 4%. These results are likewise to the reported for airlift reactors (Chisty, 1989).

For the different superficial air rates kerosene showed the highest k_La (Table 2). Although, there are no reported data for systems like these (water:kerosene emulsion and kerosene), these results are similar to reported by Subczynski and Hyde (1984) who found higher oxygen concentrations in organic compounds like sec-butyl and paraffin oil than in water.

Also, Clarke *et al.* (2006) showed a k_La enhanced up to 4-folds in a 20% alkane (n-C_{13}) – water system at 30°C, 1.5 vvm and an agitation rate in the range of 400 and 1000 r.p.m.

Oxygen supply to the microorganisms grown in the fermentors depends on the k_La. The k_La values were determined for three different air flows (Table 2). Although the highest value of k_La obtain was not very great we could hardly use better conditions for air feeding, especially for aeration rates. For the selected aeration rates is observed that the highest k_La is obtain with the highest air flow (0.425 m^3 s^{-1}).

This is an important fact, now that besides oxygen is important for *Pseudomonas* growth, it is also important for the enzymatic reaction since in the 4S metabolic pathway there are three monooxygenases involved (Gray *et al.,* 2003).

Table 1. Average and range of sulfur removal and calorific power loss for kerosene in 100 mL scale.

Microorganism	% Sulfur removal	% Calorific power loss
ATCC 39327	19.9 (19.1 – 21.2)	0.45 (0.38 – 0.48)
02	35.9 (35.7 – 36.1)	5.55 (5.11 – 5.99)
05	34.0 (32.9 – 35.7)	3.64 (2.99 – 4.28)
06	62.6 (61.8 – 63.1)	4.52 (4.26 – 4.79)

Table 2. Average k_La^* measures in airlift reactor.

Air Flow (m^3 s^{-1})	***Water***	***Kerosene***	***Emulsion (50:50)***
0.142	1.9	10.2	4.0
0.283	4.3	13.6	4.6
0.425	9.4	18.7	5.5
Maximum deviation (%)	7	6.5	5.4

*K_La (s^{-1}) *10^3

C. Sulfur Removal from Kerosene in Airlift Reactor.

Strain Nº 06 was selected for DBS assays in airlift reactor due to its high sulfur removal levels in 100 mL scale. Power capacity loss and percentage of sulfur removed were determinated after seven days culture in the airlift.

Previously, we showed that the presence of ethanolamine oleate increase near 4-fold the ATCC 39327 biodesulfurization activity respect to culture medium without surfactant (Sánchez *et al.*, 2004). Due to the ionic characteristics of ethanolamine oleate, culture medium pH modification from 7.6 to 8.0 allowed reducing its amount from 5000 ppm to 3500 pm, getting the same phase separation time. This pH modification did not have any major effect over cell viability. For both strains, the airlift reactor culture conditions were the same (air flow 0.34 m^3 h^{-1} and 30°C for 7 days).

After 7-culture days, sulfur removal for ATCC 39327 and strain Nº. 06 were 64 and 53 %, respectively (Fig. 2). It is observed a high biodesulfurization rate in the first 24h but no major changes are displayed in the last 48 h. Additional studies with ATCC 39327 have not displayed an increment in BDS activity after 12 days (data not shown). Similar results were obtained by Lee *et al*. (1995), who using a Gram-positive isolated bacterium did not enhance the sulfur removal after 8 days of culture.

The ATCC 39327 sulfur removal was 2.6-folds higher in the reactor than in the 100 mL scale. Since this is a mixed culture, it is possible that the airlift operation conditions (not presented at 100 mL scale) like aeration, gas renovation and flow pattern could improve the growth and metabolism of other microorganisms rather than *Pseudomonas* with BDS activity. On the other hand, sulfur removal with the native strain Nº 06 at reactor scale was reduced in about a 15%. This could be associated with less accurate oxygen and nutrients transport at the selected operation conditions in the reactor than in 100 mL culture. This was an expected finding, due to the

normal decrease in the mass transfer during the scale up by heterogeneities in the mixing pattern (Hewitt and Nienow, 2007). Although, further evaluation of operational conditions with strain Nº 06 are necessary to improve the sulfur removal levels; these results show the BDS potential of strain Nº 6 in an airlift reactor.

Average calorific power loss with both strains was about 20 KJ g^{-1} (about a 4,5%) (Fig 3). As it was mentioned, this reduction in the calorific power is lower than previous BDS reports (Le Borgne and Quintero, 2003). The calorific power loss in the airlift reactor cultures with strain Nº 06 were similar to the obtained results in 100 mL culture, while ATCC 39327 showed a higher calorific power loss in the airlift reactor compare to 100 mL scale. This agrees with their biodesulfurization activity. Previously, we described that ATCC 39327 and strains Nº 02, 05 and 06, use the 4S pathway for sulfur removal from DBT (Alméciga-Díaz *et al*, 2005). In this pathway sulfur is removed like sulfate, while carbon backbone remains like 2-hydroxybiphenyl (McFarland *et al*., 1998; Monticello, 2000). However, chromatographic analysis of kerosene before and after fermentation did not show DBT or its metabolites, pointing to the presence of a different pathway on these *Pseudomonas* strains to carry out the BDS process, where sulfate should be one of the end products Additionally, the calorific power loss could be caused by the generation of water-soluble end products like in the Kodoma's pathway, where sulfur is removed like 3-hydroxy-2-formyl-benzotiophene, or the use of a completely oxidative pathways to CO_2 and SO_4^{2+} (McFarland *et al.*, 1998). In the same way, the higher calorific power loss observed with ATCC 39327 in the airlift reactor could be associated to the presence of other strains than *Pseudomonas* with different metabolic pathways. This hypothesis is withstood by the higher BDS activity observed for ATCC 39327 in the airlift reactor. However, further analyses are necessary to determine if the calorific power loss has a deleterious effect over the kerosene

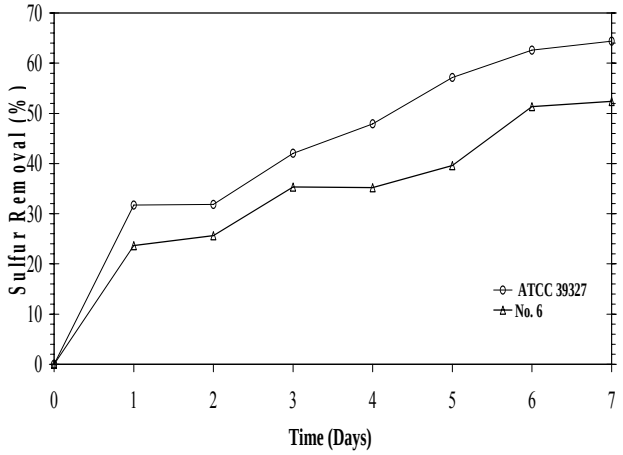

Figure 2. Sulfur removal level from Kerosene in airlift reactor by ATCC 39327 and native strain Nº 6.

Sulfur removal percentages of this work were similar to obtained using Continuos Stirred Tank Reactor designed by Energy Biosystems Corporation which permitted a sulfur removal level between 50 to 70% over diesel (McFarland *et al.,* 1998), and better than obtained with a similar system designed by Brookhaven National Laboratory which permitted a sulfur removal level between 25 to 35% (McFarland *et al*., 1998). Other bioreactors, like Fluidized Bed Reactors or Emulsion Phase Contactor (where biocatalyst is spread out in the fuel using a spray that make a fine emulsion or where biocatalyst is immobilized and fuel pass through it), are still in development and there is not data of their sulfur removal capacity (McFarland *et al*., 1998; Monticello 2000).

IV. CONCLUSIONS

Native strain Nº 06 is showed like a BDS alternative of fossil fuels due to its high sulfur removal percentage (53%), low calorific power loss (4,5%) and viability preservation. Scale-up process allowed establishing that hydrocarbon presence has a positive effect over K_La. Although there was not an increment on the sulfur removal level from 100 mL scale to airlift reactor scale, this paper show the potential use of native strain Nº 06 and airlift reactor for fossil fuels BDS.

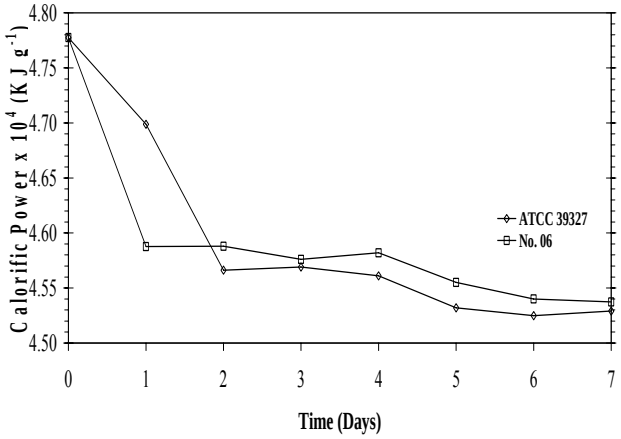

Figure 3. Kerosene calorific power in airlift reactor by ATCC 39327 and native strain Nº 06.

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