



Evaluation of Crude Oil Degradation by *Yarrowia lipolytica*

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Yarrowia lipolytica is a fungus that degrades hydrophobic substrates very efficiently. Due to its ability, *Yarrowia lipolytica* strains have been focus of bioremediation studies, being used as promising agent for treatment of contaminated areas. Bioremediation is an easy method to apply and substantially viable, among several spill control techniques. This technique involves the acceleration of natural biodegradation in contaminated environments through increasing nutrients availability and improving environmental conditions by biostimulation, and microorganism addition by bioaugmentation. In the present work, the potential degradation of *Yarrowia lipolytica* IMUFRJ 50682 is evaluated. A 2³ full factorial design was used to investigate some variables influence (agitation, temperature and C:N ratio) on crude oil biodegradation and biomass production was used as dependent variable in experimental design. Additionally, analysis of gas chromatography-mass spectrometry (GC-MS) was made to investigate the groups of organic compounds present in crude oil can be assimilated by this strain. According to results, it's possible to observe that agitation speed showed the highest significant effect on dependent variable, followed by C:N ratio. Agitation speed presented positive effect and C:N ratio presented negative effect. Temperature did not show statistically significant effect on biomass production. Analyzing results of GC-MS fingerprints, *Y. lipolytica* proved to be a microorganism with potential application for bioremediation process, being capable to consume approximately 90% of n-alkanes, 97% of naphthalenes and even 95% of phenanthrenes.

1. Introduction

Yarrowia lipolytica was earlier referred to as *Candida lipolytica* and was included in the deuteromycetous group until the sexual stage was described. With the observation of ascospores, the fungus was reclassified and renamed as *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica* and subsequently as *Yarrowia lipolytica*. *Y. lipolytica* is a fungus that degrades hydrophobic substrates very efficiently (Bankar et al., 2009). Due to its ability, these strains have been focus of bioremediation studies, being used as promising agent for treatment of contaminated areas.

Bioremediation attempts to accelerate the natural biodegradation rates through optimization of limiting environmental conditions and was show to be an economic, versatile and ecologically acceptable cleanup technology (Margesin, 2000). This technique involves acceleration of natural biodegradation in contaminated environments through increasing nutrients availability and improving environmental conditions by biostimulation, and microorganism addition by bioaugmentation (Ferreira, 2009).

The purpose of this study was investigate the influence of agitation speed, temperature and C:N ratio on crude oil biodegradation by *Yarrowia lipolytica* through 2³ full factorial design. Technique gas

chromatography-mass spectrometry was used to investigate the groups of organic compounds that can be assimilated by this strain.

2. Materials and Methods

2.1 Culture Medium

The mineral medium used had the following composition: 7 g.L⁻¹ KH₂PO₄, 2.5 g.L⁻¹ Na₂HPO₄, 1.5 g.L⁻¹ MgSO₄.7H₂O, 0.15 g.L⁻¹ CaCl₂, 0.15 g.L⁻¹ FeCl₃.6H₂O, 0.02 g.L⁻¹ ZnSO₄.7H₂O and 0.06 g.L⁻¹ MnSO₄.H₂O (Papanikolaou *et al.*, 2002). To adjust C:N ratio for desired condition was added glucose and (NH₄)₂SO₄ at mineral medium. It is not considered carbon and nitrogen presents in the crude oil to calculate C:N ratio. The crude oil used in this study was Marlim, provided by Petróleo Brasileiro S.A. (PETROBRAS).

2.2 Microorganism

Yarrowia lipolytica 583 IMUFRJ 50682 was isolated from tropical estuarine water in Rio de Janeiro, Brasil (Haegler; Mendonça-Haegler, 1981).

2.3 Experimental

2.3.1 Preservation and culture conditions

The strain was maintained at 4°C on YPD medium with 2% w/v glucose, 2% w/v peptone, 1% yeast extract w/v and 2% w/v Agar-Agar (Silva *et al.*, 2010). For inoculum, cells were cultivated at 28°C in a rotary shaker at 160 rpm using flasks of 500 mL containing 200 mL of YPD medium. After 48 hours of cultivation, these cells were centrifuged and inoculated at a concentration of 1 mg of cells (dry weight) per mL of mineral medium.

2.3.2 Study of variables influence in biodegradation process

A 2³ full factorial design was used to investigate the influence of agitation speed, temperature and C:N ratio on crude oil biodegradation. In this design, 17 experiments were performed, including three central points. The range and the levels of investigated variables are given in Table 1. It is possible to visualize the values of each variables in inferior point (-1), superior point (+1), axial points (-1.68; +1.68) and central point. Biomass production was chosen as dependent variable of the experimental design.

The experiments were performed in flasks of 125 mL containing 50 mL of culture medium and 1.0% v/v of crude oil during 96 hours. The glucose concentration was fixed at 20 g.L⁻¹, but ammonium sulfate concentration was added in different amounts to obtained the C:N ratio desired .

In parallel, an abiotic control was performed to calculate oil loss by evaporation, sampling or other systems conditions that may cause oil loss.

Table 1: Experimental range and levels of independent variables used in 2³ full factorial design for crude oil biodegradation.

Variables	Level				
	-1.68	-1	0	1	1.68
Temperature (°C)	24.5	26	28	30	31.5
Agitation speed (rpm)	60	110	180	250	300
C:N ratio	10:6.25	10:2	10:1	10:0.67	10:0.54

2.3.5 Oil remaining extraction

The oil remaining extraction was performed after 96 hours of experiment through addition of 50 mL chloroform.

2.4 Analytical Methods

2.4.1 Cell Growth Determination

Cell Growth determination was followed by optical density (O.D.) measures at 570 nm and the O.D. values were converted to cell dry weight per volume (mg dw.mL^{-1}) using a factor previously determined.

2.4.2 Statistical Analysis

“STATISTICA” (version 7.0) software was used to perform the statistical analysis of data obtained.

2.4.3 Gas chromatography-mass spectrometry analysis (GC-MS)

The oil remained from experiments were analyzed by GC-MS. GC-MS analysis were performed in Agilent Technologies gas chromatograph coupled to Agilent Technologies 5973 mass spectrometer utilizing helium as carrier gas and the following conditions: fused-silica column HP-5 (30 m \times 0.25 mm, df – 0.25 μm , J and W Scientific), 60°C–300°C at 6°C/min, held isothermal at 300°C for 20 min, splitless, temperature of injector was 290°C. Electron impact ionization at 70 eV was used. All samples were analyzed in full-scan mode and range 50-550 Dalton.

3. Results and Discussion

3.1 Study of the variables that influence the biodegradation process

The experiments realized and biomass productions obtained (ΔX) are listed in Table 2. After 96 hours of experiments, biomass production ranged from 2.66 mg.mL^{-1} to 14.89 mg.mL^{-1} . The results obtained in central point (assays 15, 16 e 17) have a standard deviation of 5%, showing that these experiments are reproducible. Furthermore, it is possible to observe that the difference between maximum and minimum values obtained for dependent variable (12.23 mg.mL^{-1}) is greater than the variation of maximum and minimum values for central points (0.98 mg.mL^{-1}). Therefore, this fact confirms that different values were obtained due to changing conditions imposed in biodegradation process.

The highest biomass production was obtained in experiment 12 that was carried out at higher speed agitation. Lower biomass production was detected in the experiment 11 that was carried out at lower agitation speed, but C:N ratio was the same as experiment 12. So, it is possible to verify that agitation speed is important parameter to biodegradation process. The assays performed at highest agitation speed (assays 3, 4, 7, 8 and 12) resulted in greater biomass production.

Table 2: Experimental conditions and results of biomass production after 96 hours of experiments.

Assays	Temperature (°C)	Agitation (rpm)	Ratio C:N	$\Delta X(\text{mg.mL}^{-1})$
1	26	110	10:2	8.29
2	30	110	10:2	8.61
3	26	250	10:2	12.30
4	30	250	10:2	12.49
5	26	110	10:0.67	9.25
6	30	110	10:0,67	9.21
7	26	250	10:0,67	13.67
8	30	250	10:0.67	12.32
9	24.5	180	10:1	11.72
10	31.5	180	10:1	9.64

11	28	60	10:1	2.66
12	28	300	10:1	14.89
13	28	180	10:6.25	11.56
14	28	180	10:0.54	3.40
15	28	180	10:1	9.71
16	28	180	10:1	9.98
17	28	180	10:1	10.69

Figure 1 shows the Pareto chart generated by experimental design performed using biomass production as dependent variable. It is possible to verify that agitation speed had the highest significant effect on dependent variable, followed by C:N ratio. Agitation speed presented positive effect and C:N ratio presented negative effect. Temperature did not show statistically significant effect on biomass production.

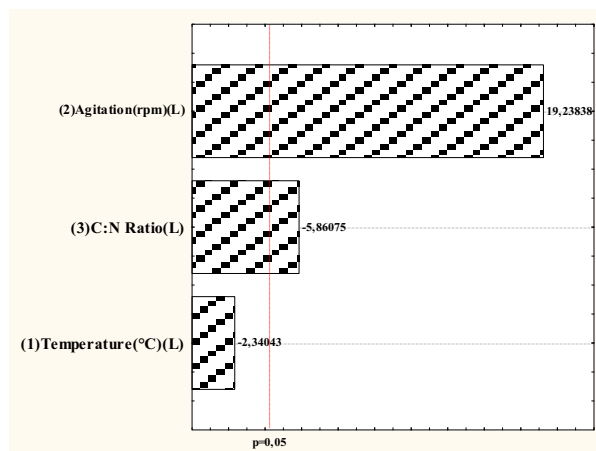


Figure 1: Pareto chart generated by Statistica 7.0 software to analyze variables influence in biomass production.

In Table 3 are listed values of each variables effect, standard deviation, value of t-student and p-level significance.

Table 3: Regression coefficients, standard deviation, t-student and p-level for each variable analysed.

	Regr. Coefficients	Deviation	t(2)	p ($\alpha = 95\%$)
Average	10.0229	0.1228	81.6398	0.0001
Temperature(L)	-0.3205	0.137	-2.3404	0.1441
Agitation speed(L)	2.6352	0.137	19.2383	0.0027
C:N Ratio(L)	-0.8028	0.137	-5.8607	0.0279

Positive effect of agitation speed on biomass production is justified because the strain used in this work is strictly aerobic. So, highest speed agitation allows increase medium aeration which contributes to cell growth.

The negative effect of C:N ratio is an interesting result that demonstrates the importance of optimization and also indicates an inadequate proportions of carbon and nitrogen in medium, which can limited cell growth. Temperature did not show statistically significant effect on biomass production. *Y. lipolytica* IMUFRJ 50682 metabolism is not significantly influenced by temperature at range from 24.5 °C to 31.5 °C. So, it is not required rigorous temperature control.

3.2 *Yarrowia lipolytica* potential degradation

Biodegradation level can be estimated quantitatively by analysis of some biomarkers. In this study, GC-MS analysis allowed to investigate these compounds.

The chromatographic fingerprint in Figure 2 shows the m/z 85 ion from abiotic control (A) and experiment 12 (B), characteristic of alkanes. The presence of *n*-alkanes, pristane and phytane is visible. Biodegradation process in experiment 12 reduced around 90% of *n*-alkanes, pristane and phytane in relation to abiotic control.

In this work, it was also investigated aromatic hydrocarbons, e.g., naphthalenes and phenanthrenes (Wiedmann, 2006).

Analyzing Figures 3(C) and 3(D), it is possible to confirm the assimilation of aromatic compounds as naphthalene and methyl-naphthalenes by *Y. lipolytica*. The abundance of these compounds in experiment 12 was approximately 97% less that in abiotic control.

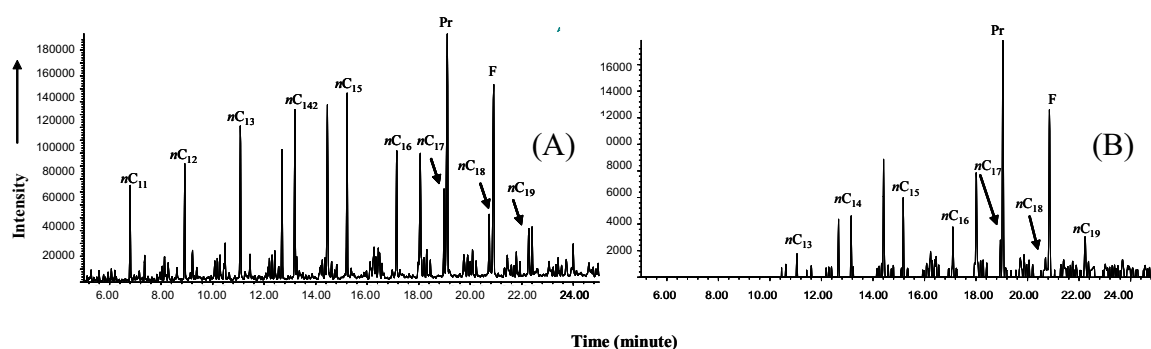


Figure 2: The chromatographic fingerprint shows the m/z 85 ion from abiotic control (A) and assay 12 (B).

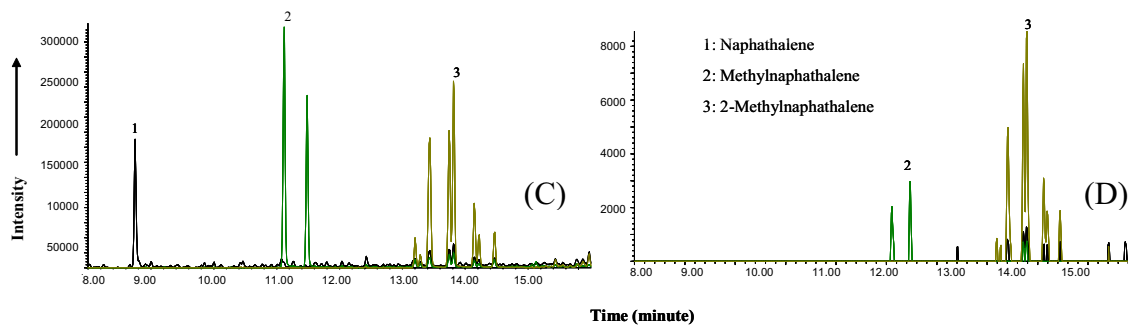


Figure 3: The chromatographic fingerprint shows the m/z 128, m/z 142 and m/z 156 ions from abiotic control (A) and assay 12 (B).

The fingerprint in Figure 4 shows the ions of phenanthrene compounds from abiotic control (E) and experiment 12 (F). Biodegradation process in experiment 12 reduced even 95% of phenanthrene compounds in relation to abiotic control.

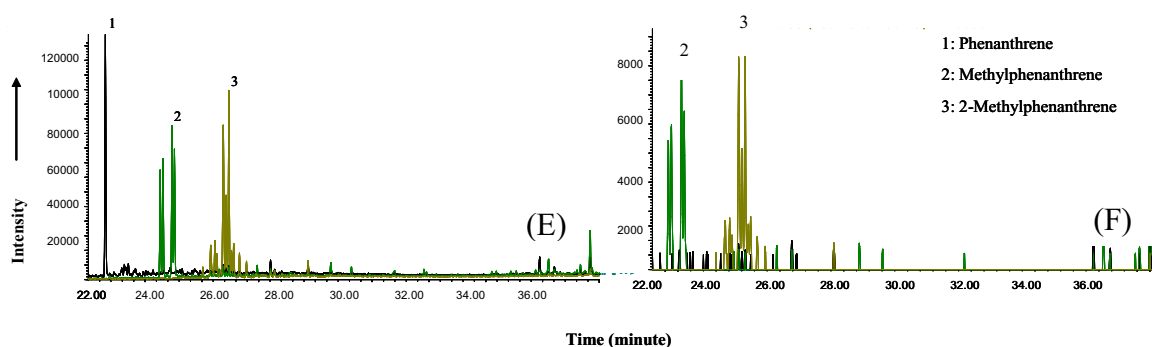


Figure 4: The chromatographic fingerprint shows the m/z 178, m/z 192 and m/z 206 ions from abiotic control (A) and assay 12 (B).

4. Conclusion

The agitation speed presented the highest effect on biomass production (ΔX), being it positive. The C:N ratio also showed statistically significant effect on biomass production, however, it was negative. Furthermore, temperature did not have statistically effect inside the range studied in this work. Therefore, can be concluded that agitation speed is the most important parameter for increases microbial population and consequently oil biodegradation.

The GC-MS fingerprints obtained confirmed that biodegradation was higher in experiments with larger biomass production. For example, in experiment 12, which had the higher biomass production, the n-alkanes degradation was 90%, the naphthalene removal was 97% and the phenanthrene remediation was approximately 95% in relation abiotic control.

5. References

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