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PRELIMINARY STUDIES ON YEAST-PLANT SYSTEMS WITH APPLICATIONS IN PHYTOREMEDIATION

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Abstract

Phytoremediation represents an ecological and economic alternative for remediation of polluted environments. The combination of plants and xenodegrading microorganisms is often used for the improvement of the remediation process. Yeast strains Yarrowia lipolytica CMGB32, Cryptococcus curvatus YR-P2 and Rhodotorula glutinis RG5 were tested for assimilation of petroleum and n-hexadecane and biosurfactant synthesis. Y. lipolytica CMGB32 grew well on n-hexadecane over two weeks, C. curvatus YR-P2 degraded petroleum during the first ten days, while R. glutinis RG5 was more active at the beginning of incubation period. All strains produced good rates of biosurfactants. Bean (Phaseolus vulgaris) seeds were sown in pots with sterilized soil and a mixture of the three yeast strains in the presence of petroleum and n-hexadecane (2:1 v:v) and observed for a month. Similar experiments were performed using oil polluted soil from oil wells (Ploiesti area, Romania) and a mixture of polluted soil, sand and gravel. The plants grew slowly on sterilized soil, with visible results only after three weeks. The presence of oil well polluted soil allowed seed germination during the first week due probably to complex interaction between soil native microorganisms-yeast mixture-plant-pollutant which provided the necessary nutrients. Although P. vulgaris has the ability to convert contaminants in less toxic compounds and to fix atmospheric nitrogen, the yeast mixture seems to enhance the plant growth, fully developed plants being observed after two weeks compared to control plants. Soil aeration was also a determinant factor, since seeds planted in soil mixture showed the most rapid growth during first week. The results suggest a positive influence over long period of time of the yeast mixture on plant growth in presence of oil compounds. Further work aims optimization of the novel yeast-plant system as basis for phytoremediation studies.

Key words*: Yarrowia, Rhodotorula, Cryptococcus, Phaseolus vulgaris, oil, phytoremediation.*

INTRODUCTION

Phytoremediation represents a promising green technology aimed to restore the environment by using the ability of higher plants to convert a wide range of pollutants (petroleum, hydrocarbons from oil spills, heavy metals, household wastes) into less toxic compounds through various metabolic pathways. Phytoremediation involves different processes: phytoaccumulation, phytostabilization, phytodegradation, phytovolatilization and rhizodegradation (Zhou et al., 2011). The possibility of applying phytoremediation *in situ*, without supplemental costs related, for example, to soil transportation and treatment *ex situ*, augmented the interest for improvement research studies. Thus, enhancement of phytoremediation by inoculation of plants with microorganisms is based on the multiple, synergic interactions between the plant root system and the microbial population (bacteria, yeasts, fungi). Yeast species associated with the rhizosphere belong mainly to *Candida krusei, Candoda maltosa, Cryptococcus curvatus, Cryptococcus laurentii, Debaryomyces hansenii, Metschnikowia pulcherrima, Pichia (Candida) guilliermondii, Rhodotorula glutinis, Saccharomyces cerevisiae* and *Yarrowia lipolytica*. The yeast population is larger in the rhizosphere than in the rest of the bulk soil, due to the presence of chemical exudates (aminoacids, carboxylic and phenolic acids, carbohydrates, mucilage and plant cell lysates) resulted from plant and fungi metabolism (Botha, 2006). On the other hand, the yeasts contribute to the carbon and nitrogen cycle in soil, increase nodulation and stimulate mychorrizal-root colonization helping to plant growth promotion (Singh et al., 1991).

Many of the yeast species from the *Candida*, *Cryptococcus*, *Rhodotorula* and *Yarrowia* genera isolated from soil are also able to degrade hydrocarbons and to synthesize

biosurfactants that can be successfully used for bioremediation of the polluted soil, including for agriculture (Sachdev and Cameotra, 2013; Shekhar et al., 2015). The biosurfactans are low molecular weight surface-active amphiphilic compounds able to reduce the tension at hydrophobic /water interfaces, being thus able to enhance the availability of hydrocarbons for biodegradative processes.

Weed plants (Wei et al., 2010) are known to have a good potential for phytoremediation due to their extensive fibrous root system, which offers a large surface to establish a strong rhizosphere in the contaminated soil. However, legume plants have been studied intensively in many countries since, besides having an extensive root system, they are also nitrogen independent (Ndimele, 2010, Mathur et. al 2010). This fact is very important in oil-contaminated soils because they do not have to compete with the endogenous microorganisms for the limited supplies of available nitrogen. Thus, the plants can grow and produce sufficient biomass assuring an efficient bioremediation (Udom et al., 2015). For example, Yateem et al. (2000) studied the effect of three economically important plants, alfalfa (*Medicago sativa*), broad bean (*Vicia faba*) and rayegrass (*Lolium perenne*), on total petroleum hydrocarbon (TPH) degradation. The results obtained showed that, even if all three species presented normal growth at 1% TPH concentration, the degradation activity was higher in leguminous plants. Previous studies showed that these species, including *Phaseolus vulgaris,* were able to grow and reduce the concentration of pollutants from contaminated soil. Moreover, their root system present differences regarding the size and number of nodules, i.e. in plants grown on contaminated soil, the nodules were less numerous and larger than in control plants (Rosado et al., 2004; Nwoko et al., 2007; Udom et al., 2015).

In the present work three yeast strains *Yarrowia lipolytica* CMGB32, *Rhodotorula glutinis* RG5 and *Cryptococcus curvatus* YR-P2 are studied regarding their ability to degrade hydrocarbons (*n*-hexadecane and petroleum) and to produce biosurfactants using these substrates as sole carbon sources. Preliminary phytoremediation studies are performed comprising treatment of *Phaseolus vulgaris* with the three yeast strains and evaluation of environmental conditions influence on plant growth in oil polluted soil.

MATERIALS AND METHODS

1. Biological materials

The yeast strains *Yarrowia lipolytica* CMGB32 (Csutak et al., 2015), *Rhodotorula glutinis* RG5 and *Cryptococcus curvatus* YR-P2 from oil-polluted environment (Csutak et al., 2012; Corbu et al., 2016) were maintained in the Collection of Microorganisms of the Department of Genetics, Faculty of Biology, University of Bucharest, Romania (CMGB) on Yeast Peptone Glucose Agar (YPGA) medium (0.5% yeast extract, 1% peptone, 0.2% glucose, 2% agar-agar).

Bean (*Phaseolus vulgaris*) seeds (20 g) were sterilized with 4% sodium hypochlorite solution, for 20 min. with permanent stirring, and after the hypochlorite was drain out, the seeds were washed four times with distilled water and placed on a sterile surface till fungal inoculation (Jones, 2017).

2. Assessment of hydrocarbon biodegradation

The yeast inoculum (0.3 X 10^6 cells/ml) was cultivated in Bushnell-Haas mineral medium (KH_2PO_4 1 g/l, K_2HPO_4 1 g/l, NH_4NO_3 1 g/l, $MgSO_4 \times 7H_2O$ 0.2 g/l, $FeCl_3$ 0.05 g/l, $CaCl_2 \times 2H_2O$ 0.02 g/l) supplemented with 1% *n*-hexadecane (Sigma), respectively, petroleum (Fluka). Yeast cultures were incubated for 14 days at 28^{o}C, 120 rpm. Samples were collected at time 0 and after 2, 6, 10 and 14 days. The biodegrading abilities of the yeast strains were determined by monitoring cell counts on Thoma counting chamber and the pH (de Cassia Miranda et al. 2007; Pirôllo et al., 2008).

3. Biosurfactants production. Determination of the emulsification activity

The yeast strains were cultivated for 72 h at 150 rpm, in 20 ml Yeast Peptone (YP) medium (1% yeast extract, 1% peptone) supplemented with 1% *n*-hexadecane (Sigma-Aldrich) for *Y. lipoytica* CMGB32 and *R. glutinis* RG5 and 1% petroleum (Fluka), respectively, 1% *n*-hexadecane (Sigma-Aldrich) for *C. curvatus* YR-P2.

After 72 hours, the production of biosurfactants was evaluated using the emulsification index (E_{24}%) (Cooper and Goldenberg, 1987). Thus, cell-free broth from yeast cultures grown on biosurfactant production media were mixed (3:2 v:v) with *n*-hexadecane for the cultures grown on petroleum and with petroleum for the *n*-hexadecane grown cultures. The mixture was vortexed for 2 min at 2500 rpm and maintained at room temperature for 24 h before calculating the E_{24}%.

4. Phytoremediation assays

Three parallel phytoremediation assays were performed in duplicates, in plastic pots, in lab conditions (at room temperature, and natural illumination) and observed over four weeks.

First, two bean seeds were sown in pots with sterilized soil as follows: MI Pp – reference pot with bean seeds and YNB medium (0.67% YNB with aminoacids and ammonium sulphate, Sigma) supplemented with 1% petroleum and *n*-hexadecane (2:1 v:v), SI Pp - sample pot with bean seeds, yeast inoculum (6 x 10^5 cells/g soil) represented by a mixture of *Y. lipoytica* CMGB32, *R. glutinis* RG5 and *C. curvatus* YR-P2 in YNB medium and the same proportion of hydrocarbons (after Boby et al., 2008). The first day, the plants were wattered with YNB medium supplemented with hydrocarbonate substrate for allowing yeast growth, after which they were wetted uniformly with tap water.

In a second experiment, two bean seeds were sown in pots with oil polluted soil from oil wells (Ploiesti area, Romania): M - reference with bean seeds, P_{3+} - sample with bean seeds and yeast mixture (6 x 10^5 cells/g soil) in sterilized water.

Finally, we used a mixture of polluted soil, sand and gravel (2:1:1), where M Bis – the reference and P2 – the sample were prepared similar to the conditions from the second experiment. For both experiments the plants were wetted only with taped water.

RESULTS AND DISSCUSSIONS

Hydrocarbon assimilation

The yeast strain *C. curvatus* YR-P2 showed similar growth profiles on both hydrocarbonate substrates (Figure 1A). The *n*-hexadecane was rapidly assimilated during the first two days of incubation (1.7 X 10^6 cells/ml) after which the curve registered a descending profile until day ten. The ascending profile observed till the end of incubation period, might be due to the presence of secreted metabolites in the medium, which can be used as secondary substrates for cell growth. The petroleum, although stimulated the cell growth, was assimilated during a longer period of time, within a week (1.4 X 10^6 cells/ml), after which the cell multiplication decreased constantly.

This study is one of the few studies describing the assimilation of hydrocarbons by a *C. curvatus* strain. Thus, Sietmann et al. (2002) reported *C. curvatus* strains able to biodegrade biarylic compounds (biphenyl, dibenzofuran and diphenyl ether) producing mainly monohydroxylated intermediates as well as ring cleavage product. This might explain the good ability of *C. curvatus* YR-P2 to grow on petroleum containing approximately 80% aromatic hydrocarbons. Another explanation might reside in the fact that our strain was isolated from oil-polluted soil, the cells presenting adaptive metabolic pathways for consuming various classes of oil compounds.

Rhodotorula species, including *R. glutinis*, have been described as being able to degrade *n*-alkanes (Trama et al., 2014), aromatic compounds (Boşça and Sanin, 2015) and complex hydrocarbonate substrates such as petroleum and diesel (Shailubhai et al., 1984; de Cassia Miranda et al., 2007). The strain *R. glutinis* RG5 doubled the cell number (0.6 X 10^6 cells/ml) between the second and the sixth day of incubation on *n*-hexadecane, after which a drop was observed to the initial level and the plateau installed (Figure 1B). No growth was recorded when petroleum was used.

Y. lipolytica is a well known consumer of hydrocarbons, including *n*-alkanes (Fickers et al., 2005). The strain *Y. lipolytica* CMGB32 showed a high rate of *n*-hexadecane assimilate. The cell number tripled within only two days (1.0 X 10^7 cells/ml) and continued the accelerated growth until the end of the experiment reaching 1.1 X 10^8 cells/ml (Figure 1C).

As in the case of *R. glutinis* RG5, no growth was recorded on petroleum as sole carbon source. However, previous studies showed that *Y. lipolytica* CMGB32 produced biosurfactants in the presence of petroleum in YP medium

(Csutak et al., 2015). Since the assimilation tests were performed using a simple mineral medium (Bushnell-Haas), the results indicate the major impact of complex nitrogen, aminoacids and vitamin resources from the YP medium (yeast extract, peptone), on the cell growth, biosurfactant synthesis and, therefore, on hydrocarbon assimilation.

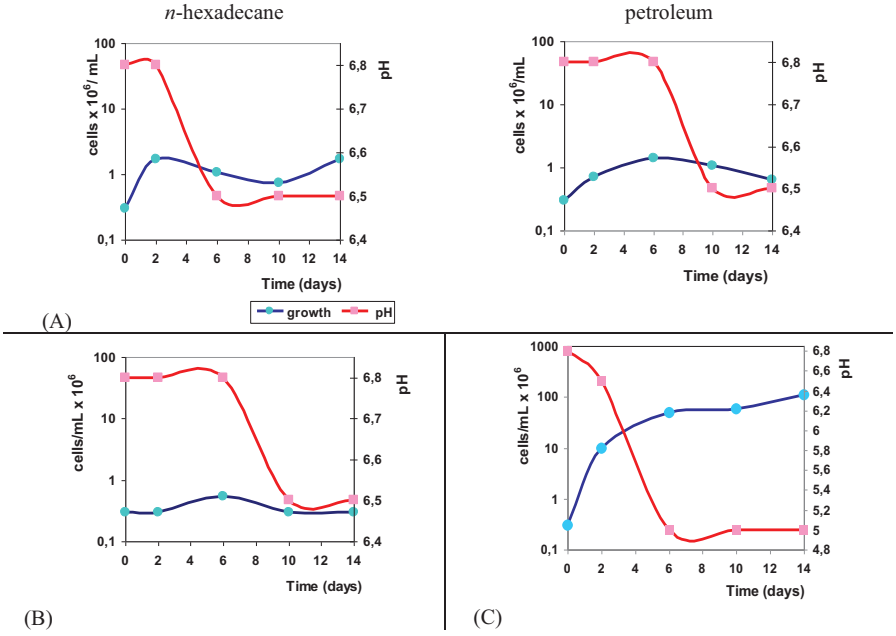

Figure 1. Growth curves and pH variation for *C. curvatus* YR-P2 (A), *R. glutinis* RG5 (B) and *Y. lipolytica* CMGB32 (C) over 14 days biodegradation of the hydrocarbonate substrates

Biosurfactant synthesis. Mechanism of hydrocarbon assimilation

Yeasts can assimilate hydrocarbons in pseudo-solubilized form due to production of biosurfactants. In the present study, we analyzed the synthesis of biosurfactants using as sole carbon source hydrocarbonate substrates for which we obtained the best growth rates for each of the three yeast strain tested.

Until present, there are few data on production of biosurfactants, respective sophorolipids, from *C. curvatus* (Daniel et al., 1999; Banat et al., 2014). The strain *C. curvatus* YR-P2 showed good growth rates both on *n*-hexadecane and petroleum. Therefore, the production of biosurfactants was tested using YP medium supplemented with each of the two hydrocarbonate substrates. The E_{24} values were 44% when grown on petroleum and 39% when *n*-hexadecane was used as sole carbon source.

Previous studies mentioned the fact that low pH values could indicate an intensive metabolism of the hydrocarbons in the cells leading to excretion of fatty acids into the extracellular medium (Oboh et al., 2006; Pirôllo et al., 2008). Meanwhile, high pH values could be related to biosurfactant production activity (de Luna et al. 2009). By correlating our E_{24} results with the data from Figure 1A, we can conclude that in the case of *C. curvatus* YR-P2, the hydrocarbonate substrates can be assimilated both throughout a passive process as well as in a pseudo-soloubilized form. Thus, the *n*-hexadecane is internalized in the yeast cells rapidly within two days, using both mechanisms which results in high growth rates, a rapid production of fatty acids and low pH after only six days. On the contrary, during the same period of time, the petroleum seems to be assimilated more slowly, mainly due to biosurfactant synthesis.

After 72 hours on YP medium with *n*-hexadecane, *R. glutinis* RG5 produced biosurfactants with an E_{24} of 33%, which represented the main mechanisms for assimilating the hydrocarbon in the cell (Figure 1B). *R. glutinis* strains were described as producing biosurfactants with high emulsification activity

when grown for 72 hours on cassava starch broth and yeast extract (Oloke and Glick, 2005) and on motor oil and burning waste vegetables (Yuri Max et al., 2012), while the same strain RG5 produced emulsification of petroleum (43%) on YP medium supplemented with *n*-decane (Csutak et al., 2012).

For the strain *Y. lipoytica* CMGB32 we obtained 52% emulsification activity in the presence of *n*-hexadecane after 72 hours, which is comparable to the results of Cirigliano and Carman (1985). In general, for *Y. lipolytica* are recommended longer incubation periods up to 144 hours in the presence of *n*-hexadecane and ground-nut oil refinery residue (Rufino et al., 2007; Amaral et al., 2008) while good biosurfactant rates were obtained after 72 hours using YP medium and petroleum (Csutak et al., 2015) or mineral medium with soybean oil refinery residue, glutamic acid and yeast extract (Rufino et al., 2008).

Observing the Figure 1C, the main mechanisms of *n*-hexadecane assimilation for *Y. lipolytica* CMGB32, seems to be based initially on biosurfactant synthesis, followed by a passive mechanisms, with a significant production of fatty acids and a drop of the pH.

Phytoremediation studies

The preliminary phytoremediation studies involved corroborated evaluations of plant growth in the presence of various hydrocarbonate substrates in the presence or absence (reference pots) of a yeast mixture. *P. vulgaris* seeds sown in sterilized soil grew slowly, even though we used a mineral medium (YNB) supplemented with hydrocabons as carbon source for allowing yeast multiplication at the beginning of the experiment. Visible plants were observed within three weeks and no further changes appeared afterwards (Figure 2). Moreover, no significant difference could be noted between the reference (MI Pp) and the plants treated with the yeast mixture (SI Pp).

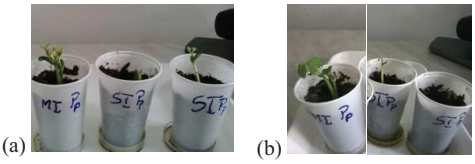

Figure 2. *P. vulgaris* seeds growth on sterilized soil with yeast mixture and added hydrocarbons after (a) three weeks and (b) one month

The situation changed when oil well polluted soil was used. After a week, the seeds already germinated and after only another six days, fully grown plants were observed (Figure 3a, b). The rapid evolution continued until the third week after which slowed down.

The influence of the yeast mixture on the plant development was obvious beginning with the third week. Thus, if within the first fourteen days, the reference plants (M) grew better than those treated with yeasts (samples P_{3+}), during the third week the sample plants showed an accelerated growth surpassing the reference (Figure 3c). This could be explained by the adaptive response of the yeasts to the environmental conditions correlated with an intense metabolism.

On the other hand, the complex chemical composition of the oil well compared to the mixture of petroleum and *n*-hexadecane used on the sterilized soil, is also expected to contribute to the yeast metabolism during this second experiment. At last, but not at least, we must also consider the complex interaction between soil endogenous microorganisms - yeast mixture – plant – complex pollutant which provided the necessary nutrients for the rapid plant growth.

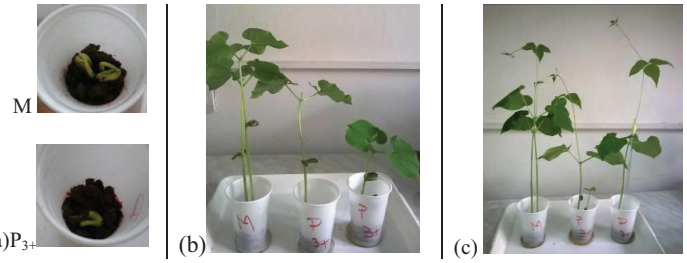

Figure 3. *P. vulgaris* seeds growth on oil well polluted soil and yeast mixture after (a) one week, (b) two weeks and (c) three weeks

Results even more spectacular were obtained when the oil well polluted soil was mixed with sand and gravel. In this case, a fast growth was observed within the first week (Figure 4).

Figure 4. *P. vulgaris* seeds growth after one week on oil well polluted soil mixed with sand and gravel in the presence of yeast mixture

This was most probably due a better aeration of the soil, an important factor both for microbial assimilation of hydrocarbons and for plant growth, since it seems that the porosity of the soil is related to changes in the diversity of the existing microbial communities (Crawford et al., 2012). However, by the end of the period of time, the development of the plants was rather similar to the previous experiment.

The results obtained during the preliminary tests, suggest a possible future modification of the yeast inoculum used for promoting *P. vulgaris* growth. Similar studies showed that a rate of 1 X 10^5 or 2 X 10^5 cells per gram of soil, could be more appropriate (Sampedro et al., 2004). Also, since all three yeast strains, *Y. lipolytica* CMGB32, *R. glutinis* RG5 and *C. curvatus* YR-P2, are good biosurfactant producers, a combination of yeast cultures and biosurfactants might assure a better hydrocarbon assimilation and plant development (Zhou et al., 2011).

CONCLUSIONS

All three yeast strains were able to assimilate *n*-hexadecane with highest rates for *Yarrowia lipolytica* CMGB32, while *Cryptococcus curvatus* YR-P2, isolated from oil-polluted soil, showed complex metabolic abilities and similar growth profiles on *n*-hexadecane and petroleum. Good results were also obtained for biosurfactant synthesis. The preliminary phytoremediation studies suggest that hydrocarbon degradation by the three yeast strains had an important positive influence on *P. vulgaris* growth over long periods of time in presence of oil compounds. The newly established yeast-plant system will be further optimized for improvement of biodegradation process and plant growth, in order to enhance the phytoremediation process.

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