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Technological Properties of Yeast Produced Surface Active Compounds in Connection with Their Application in Clean-Up Technologies

Kristina Lhotska[1, 2], Marek Sir[2], Jiri Mikes[1], Zuzana Honzajkova[2] and Miroslav Minarik[1]

1. EPS Biotechnologie, Ltd., VPastouškách 205, Kunovice 686 04, Czech Republic

2. Institute of Chemical Technology Prague, Technická 5, Praha 160 00, Czech Republic

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Abstract: Biologically produced surfactants (SACs) can mobilize and solubilize non-aqueous phase liquids (NAPL) adsorbed onto soil constituents. The interest in microbial surfactants has increased during recent years due to their lower toxicity, higher biodegradability, selectivity and specific activity under extreme conditions than synthetic SACs. Main output of the project represents preparation of this yeast biosurfactant intended for washing of matrices contaminated by NAPL. The influence of cultivation media composition on biosurfactant production was studied and basic properties (critical micelle concentration (CMC), minimum surface tension) of isolated biosurfactants were compared with properties of synthetic surfactant with surface tension measurement. The interfacial tension of the systems containing aqueous solutions of different concentrations and non-polar substances was measured with petroleum compounds (kerosene Jet A-1), aromatic and aliphatic hydrocarbons (represented by toluene and hexane). The solution of biosurfactant *Yarrowia lipolytica* (YAR) in the concentration range of 0-500 mg/L reduced interfacial tension by 80% in all representative systems with model contaminants; biosurfactant *Candida bombicola* (CAN) was less efficient. Solubilization properties were proved with toluene and hexachlorocyclohexane (HCH) isomers alpha and gamma, and effective concentration of biosurfactants was determined as 100 mg/L for toluene and HCH. SACs produced by lipophilic yeast with non-toxic and non-pathogenic status (*Yarrowia lipolytica*, *Candida* sp., etc.) seem to be very promising. The results obtained will be used for the application of biosurfactants in the clean-up technologies as agents for the mobilization of non-polar contaminants as well as for stimulation of bioremediation processes.

Key words: Biological surfactant, lipophilic yeast, carbon source, soil washing.

1. Introduction

Soil and water contamination by non-polar substances is a serious ecological and technological problem. The formation of the phase boundary is the main reason in heterogeneous environments, especially in liquid-liquid systems. However, the issue includes also different types of wastes, e.g., excavated sediments or remediation rubbles. The application of the surface active compounds (surfactants—SACs) is a solution for those types of contamination. The use of synthetically produced surfactants is the most common technology applied. Lately a growing emphasis is put on minimizing the load caused by large amounts of these substances released to the environment. Their strong persistence and negative effects on biological membranes is the reason.

Surfactants are amphiphilic molecules with both hydrophobic and hydrophilic moieties. Their important qualities include their ability to decrease a surface tension on an interface of polar and non-polar phase by accumulation on a phase boundary of two immiscible substrates, resulting in increased solubility of non-polar substances. This ability is used for instance in

Corresponding author: Kristina Lhotska, project manager, research fields: environmental microbiology and biosurfactants. E-mail: kristina.lhotska@epssro.cz.

remediation technologies. Surfactants can mobilize and solubilize non-aqueous phase liquids adsorbed onto soil constituents. Majority of surfactants currently in use are chemically derived from petroleum. However, many microorganisms are capable of producing SACs in order to overcome a phase boundary between non-polar carbon source and aqueous environment. Interest in microbial surfactants has increased in recent years due to their lower toxicity, higher biodegradability, selectivity and specific activity under extreme conditions (pH, temperature and salinity), which reduces their environmental concern [1]. The biggest obstacle of the potential application of biological SACs is their high production costs. However, critical micelle concentration of many biosurfactants is much lower than of synthetic surfactants, therefore lower surfactant concentrations can be used. Biosurfactants can provide more cost-effective approach for subsurface remediation. Moreover, they can be produced from renewable raw materials, such as waste oils [2].

The majority of microbial surfactants currently studied are of bacterial origin. The application of these compounds is restricted due to the pathogenic nature of many producing microorganisms. The study of biosurfactants produced by microorganisms with generally regarded as safe (GRAS) status has increased interest. Many non-pathogenic yeast species, such as *Yarrowia lipolytica*, *Candida* sp., *Saccharomyces cerevisiae*, *Debaryomyces* sp. and *Rhodotorula* sp., are capable of producing their own biosurfactants in order to utilize water immiscible substrates [3].

Biosurfactant molecules are divided into many groups according to the structure of their hydrophilic part, which is usually consisted of aminoacids, peptide cations or anions or carbohydrates. Hydrophobic part is formed by saturated or unsaturated fatty acids [4, 5].

Based on preliminary screening, *Yarrowia lipolytica* (yeast strain from the EPS collection of microorganisms, isolated from petroleum phase, identified by Culture Collection of Yeasts, Slovak Academy of Sciences) and *Candida bombicola* (ATCC 22214) were chosen for further experiments as biosurfactant-producing yeasts. The research is focused on application of the yeast biosurfactants in clean-up technologies, therefore it was necessary to determine properties of biosurfactants isolated from the cultivation of both yeast strains, such as critical micelle concentration (CMC), surface tension, interfacial tension and solubilization properties in aqueous solutions of biosurfactant and model non-polar contaminants.

2. Materials and Methods

2.1 Cultivation, Isolation and Purification

The stimulation of the biosurfactant production was examined with different media composition (especially carbon source). Cultivations were carried out at a cultivation temperature 25 °C. Intensive aeration was ensured and pH was not regulated during the experiments. The use of different carbon sources influences the biosurfactant production, structure and properties, and was studied. During the batch experiments, media with different carbon and nitrogen sources were tested.

After cultivation medium with biomass was put into the ultrasonic bath for 15 min to disintegrate the cells and release the biosurfactant outside from the cells to the medium. Biosurfactant produced by *Yarrowia lipolytica* (YAR) and *Candida bombicola* (CAN) in such media was later isolated by triple extraction of the broth with cells into ethyl acetate (volume ratio 3:1 was used) and then purified with hexane.

2.2 Microscopy

Vital staining with methylene blue dye enables observation of morphology of the yeast cells and viability at the same time. Culture (20 L) is dispensed on a glass slide, allowed to dry, fixed with a flame and then methanol (-18 °C) for 5 min. After adding 5 L of methylene blue, observation is

performed at a magnification of 40 10 and 100 10 by phase contrast (microscope Nikon Eclipse H 50i).

2.3 Surface Tension

Surface tension in aqueous YAR solution was measured for biosurfactant YAR, biosurfactant CAN and synthetic surfactant Reoclean for concentration range from 0 mg/L to 500 mg/L. Surface tension was measured with tensiometer K6 (Kruss GmbH) according to Du Noüy ring method. The method involves slowly lifting a ring which is often made of platinum, from the surface of a liquid. The force required to raise the ring from the liquid's surface was measured and related to the liquid's surface tension. From dependence of the surface tension on YAR concentration, a minimum surface tension and CMC were determined. CMC was calculated as an intersection of the regression lines.

2.4 Interfacial Tension

Interfacial tension of the systems containing aqueous solutions of different YAR concentrations and non-polar substances was measured by the same method (Du Noüy ring method). A ring was lifted slowly from the interface of the surfactant solution and non-polar liquid. Three representative non-polar compounds were chosen. Aromatic hydrocarbons were represented by toluene, aliphatic hydrocarbons by isooctane and petroleum compounds by kerosene Jet A-1.

2.5 Solubilization Tests

Solubilization tests were carried out with working solutions of biosurfactant in a concentration range of 0-500 mg/L. Toluene and hexachlorocyclohexane (HCH) were analyzed by gas chromatography with mass spectrometric detection.

2.5.1 Solubilization with Toluene

20 mL of biosurfactant solution was dosed in flasks and then an excess of toluene was added (1 mL). Flasks were shaken on the orbital shaker at 100 rpm for 72 h. Then the conical flask was left at room temperature for 72 h in order to complete phase separation. The aqueous phase (solution of surfactant with solubilized toluene) was then extracted with pentane in vial in a volume ratio of 1:10 for 15 min. The extract was diluted and analyzed.

2.5.2 Solubilization with Technical HCH (Isomers of Alpha, Beta, Gamma, Delta and Epsilon)

From each biosurfactant solution, 50 mL was dosed in Erlenmeyer flasks and 10 mg HCH was added. It provided the sufficient excess of HCH. Flasks with solutions were shaken on the orbital shaker at 100 rpm for 72 h and the mixture was allowed to settle down for 24 h. Subsequently, 20 mL of the solution and 10 mL of hexane were extracted by intensive shaking for 15 min. The extract was then separated and the concentration of HCH in the organic extract converted to the concentration of HCH in surfactant solution was determined. Furthermore, the weight solubilization ratio of HCH and surfactant was determined.

3. Results and Discussion

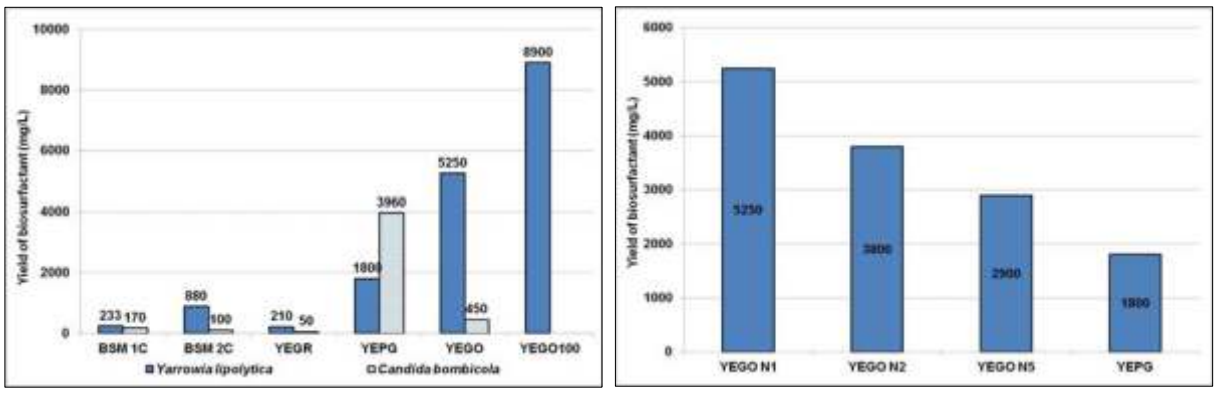
3.1 Cultivation Media Composition

The most underlying factor influencing biosurfactant production is carbon source [5, 6]. Several media which were different in composition were tested to get knowledge about optimal proportion of the components of the cultivation medium. Different carbon sources were used (sunflower oil, crude oil and kerosene) to evaluate the optimal one. Also media with one or two carbon sources (one hydrophilic and the second lipophilic) were used. The composition of cultivation media is shown in a Table 1.

Results are summarized in Figs. 1a and 1b. Best yields were obtained when culture media contained both hydrophilic and lipophilic carbon source (glucose and sunflower oil). Sunflower oil as a lipophilic carbon source is the best for yeast to utilize. In media with crude oil (YEGR), the yields of biosurfactant were much lower and the vitality of the cultivation was not satisfactory as well. Both yeast species show

Table 1 Cultivation media composition.

Ingredients (g/L)	Cultivation media					
	BSM 1C	BSM 2C	YEGR	YEGK	YEPG	YEGO
Sunflower oil	10	10	-	-	10	10
Crude oil	-	-	10	-	-	-
Kerosene	-	-	-	10	-	-
Glucose	-	10	10	10	10	100
BSM	Present	Present	-	-	-	-
Yeast extract	-	-	1	1	1	1
Peptone	-	-	-	-	2.5	-



(a) (b)

Fig. 1 Biosurfactant yields by the media composition: (a) carbon source and nutrients; (b) nitrogen source dosage (comparison of *Yarrowia lipolytica* and *Candida bombicola*).

almost no growth in media with kerosene (YEGK) as a carbon source, therefore extraction of those media was not performed.

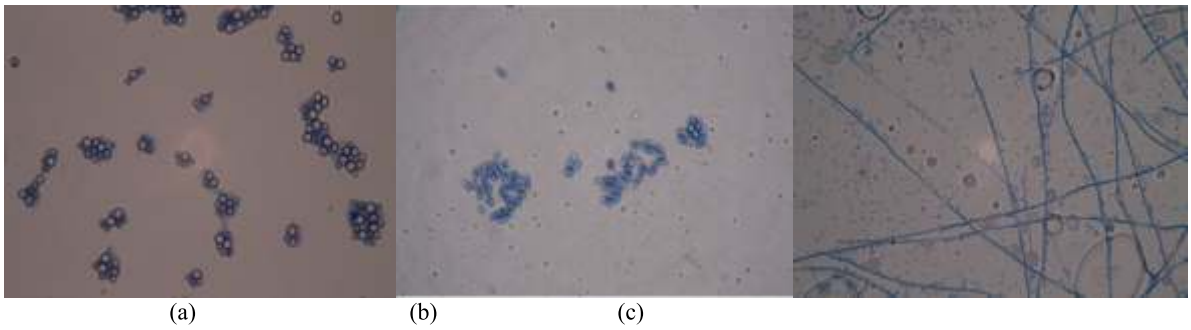
Cultivation media composition experiments evaluated yeast extract as the optimal nitrogen source for biosurfactant production. The aim of further experiments was to determine the optimal dosage. For this purpose a test with four media was prepared, based on the composition of YEGO10.

Overview of the proceeds of the experimental cultivation is shown in Fig. 1b. The lowest dosage of yeast extract (YE, 1 g/L) as a nitrogen source is the most appropriate. The highest yields were obtained at the concentration of YE 1 g/L. Yields declined with increasing doses of yeast extract or peptone addition, respectively. Considering the operational applications, the result is very promising.

3.2 Morphology

Cells of *Candida bombicola* are coffee been-shaped (Fig. 2a) and have only one morphologic form, even when changing the pH. Cells of *Candida bombicola* do not form pseudomycelium or true mycelium. In contrast, *Yarrowia lipolytica* cells are usually elongated or sickle shaped (Fig. 2b). The conditions, that induce the dimorphic transition of yeast-to-mycelium or vice versa are very variable (temperature, atmosphere of growth). The most important factors are pH or presence of specific compounds in cultivation medium, especially glucose [7]. In common media, *Yarrowia lipolytica* grows as a mixture of yeast-like and short mycelial cells. Probability of mycelium formation is maximal at near neutral pH and decreases when pH lowers from three to almost zero. Mycelial growth is also favored, when glucose in cultivation medium is present in high concentration (Fig. 2c).

Conditions that affect dimorphism of *Yarrowia lipolytica* depend on the strain used [8]. *Yarrowia lipolytica* used in this work showed mycelium formation



(a) (b) (c)

Fig. 2 Vital staining by methylene blue—*C. bombicola* cells (a), *Y. lipolytica* cells ((b) yeast-like; (c) mycelial).

when pH was lower than 6, but this pH level never decreased under 3.5. No direct effect of glucose on mycelial or yeast-like form was observed. Mycelium formation in those cases was probably caused by anaerobic stress or constant supply of carbon source. When the carbon source-substrate was supplied more often than yeast needed, yeast started to accumulate the substrate in its cells and stopped to utilize the rest of the substrate. The activity and vitality of the cells decreased and mycelium was formed. At the same time, production of surface active compounds was practically stopped. This fact was proved by oil spreading assay [9], when no clear zones were formed and the biosurfactant yields of extraction were nearly zero. It was therefore concluded that biosurfactant is formed especially when the yeast *Yarrowia lipolytica* grows as individual cells. Although microscopic control of cultivation is an indirect method, it is very important tool for the evaluation of the biosurfactant production process.

3.3 Surface Tension

Surface tension dependence on biosurfactant concentration is shown in Figs. 3 and 4. Minimum surface tension was determined and critical micelle concentration was calculated.

Properties of biosurfactants and synthetic surfactants were compared by determination of fundamental parameters of synthetic surfactant Reoclean (Fig. 5), which is commonly used for soil washing. The commercial product also contained surfactants in the form ethoxylated fatty alcohol (max. 10%), KOH (max. 1.9%) and nitrilotriacetic acid (max. 0.5%).

Surfactants with lower values of the CMC and minimum surface tension are more suitable for remediation applications than those with higher values. CMC value and minimum surface tension of Reoclean (Fig. 5) was lower than those of both biosurfactants. On the other hand, high pH value of Reoclean (pH 12) causes significant environmental impact of this surfactant. The value of the CMC was also significantly affected by the presence of even small amounts of impurities. In case of pure surfactants (e.g., Reoclean), second regression line directive is zero and the line is parallel with x-axis. In case of yeast biosurfactants YAR and CAN, second regression line slope is probably caused just because of impurities. Basic properties of biosurfactants depend mainly on cultivation conditions. Pure product could be obtained by column chromatography with activated silica gel as a stationary phase.

3.4 Interfacial Tension

Interfacial tension of the systems containing aqueous solutions of different YAR concentrations and non-polar substances was measured. In this study, three representative non-polar compounds were chosen. Petroleum compounds were represented by kerosene Jet A-1, aliphatic hydrocarbons by hexane and aromatic hydrocarbons by toluene. Decrease of interfacial tension in the solution of YAR and CAN was measured and compared for those two biosurfactants (Figs. 6-8).

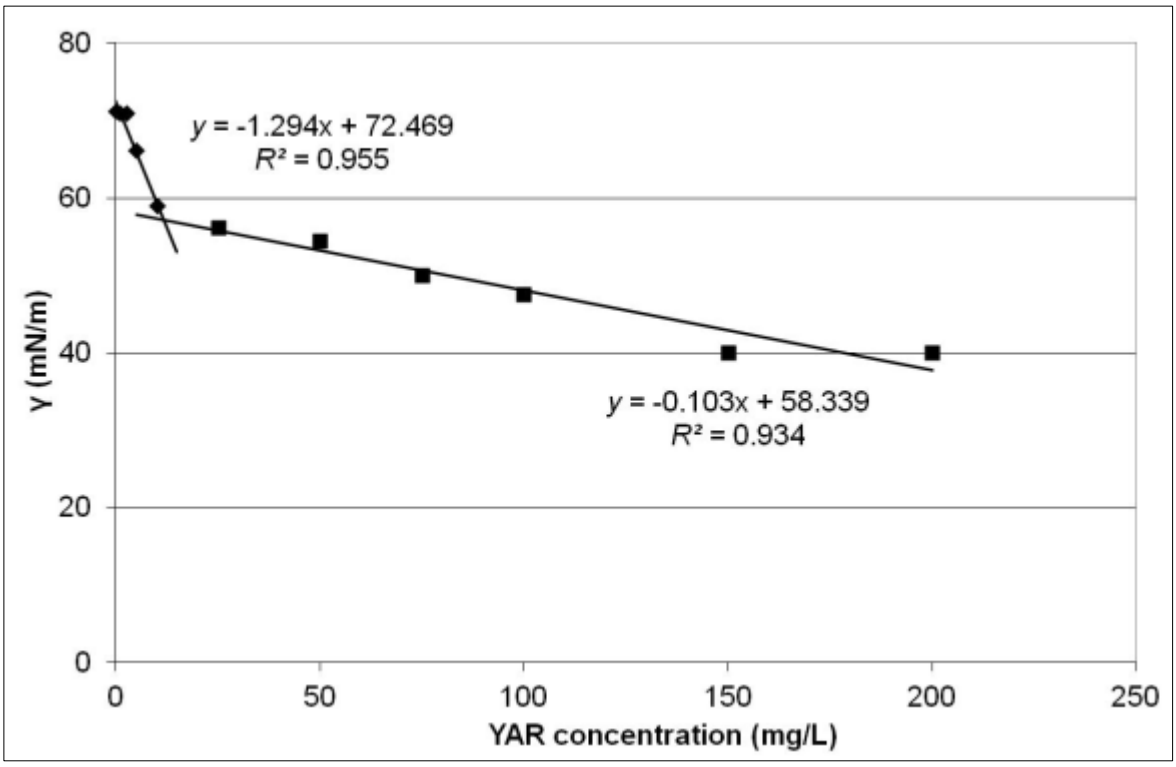


Fig. 3 Dependence of surface tension of the surfactant solution YAR on its concentration, including regression lines. Critical micelle concentration of YAR was 12 mg/L, minimum surface tension 40 mN/m.

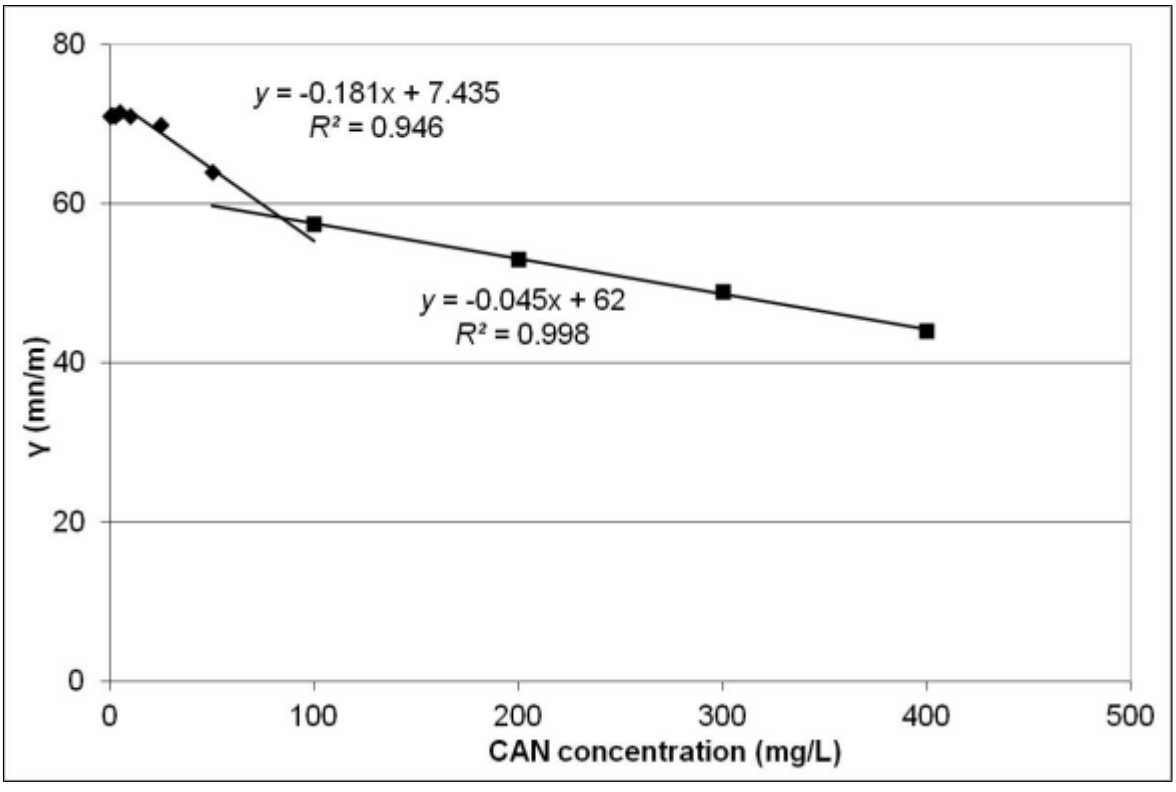


Fig. 4 Dependence of surface tension of the surfactant solution CAN on its concentration, including regression lines. Critical micelle concentration of CAN was 84 mg/L, minimum surface tension 44 mN/m.

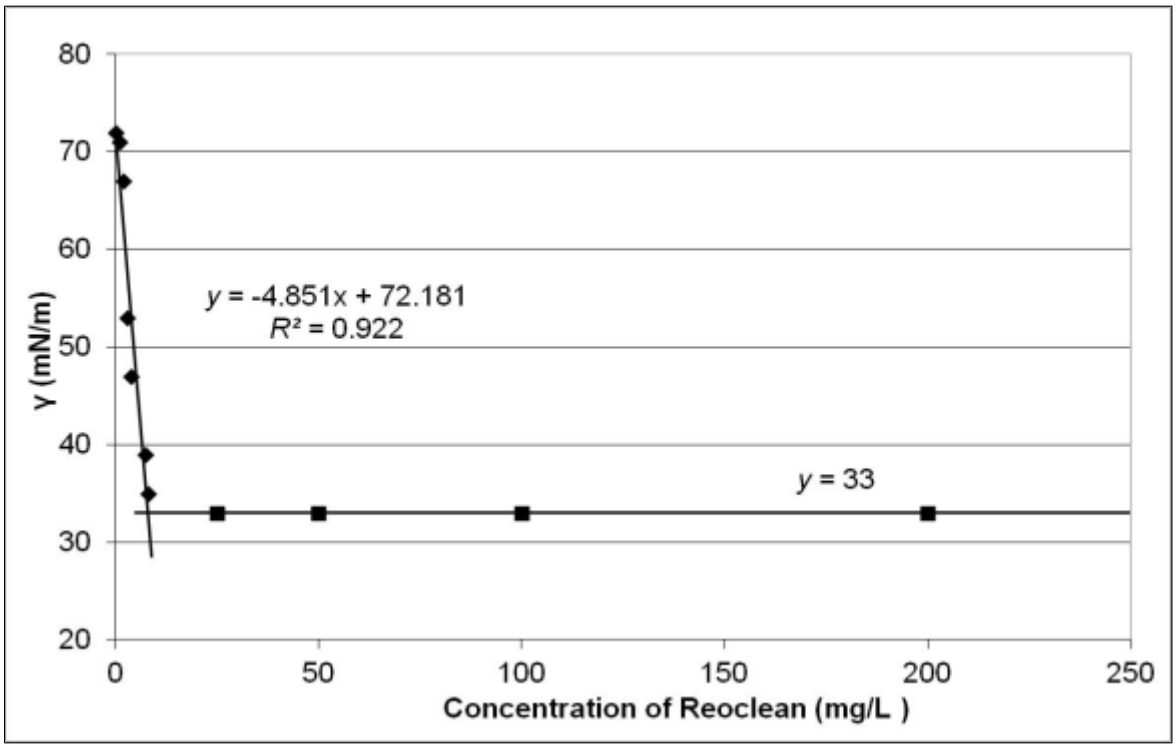


Fig. 5 Dependence of surface tension of the surfactant solution Reoclean on its concentration, including regression lines. Critical micelle concentration of synthetic surfactant Reoclean was 8 mg/L, minimum surface tension 33 mN/m.

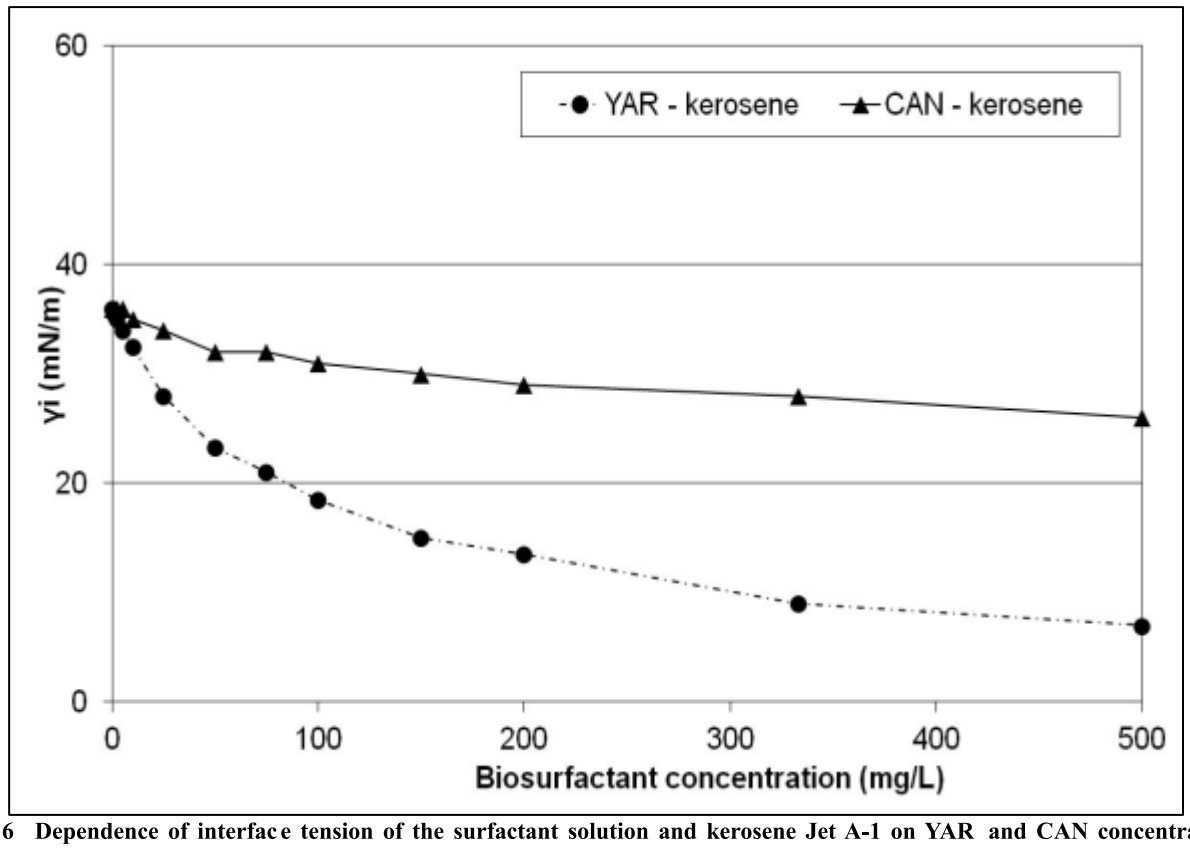


Fig. 6 Dependence of interface tension of the surfactant solution and kerosene Jet A-1 on YAR and CAN concentration (comparison).

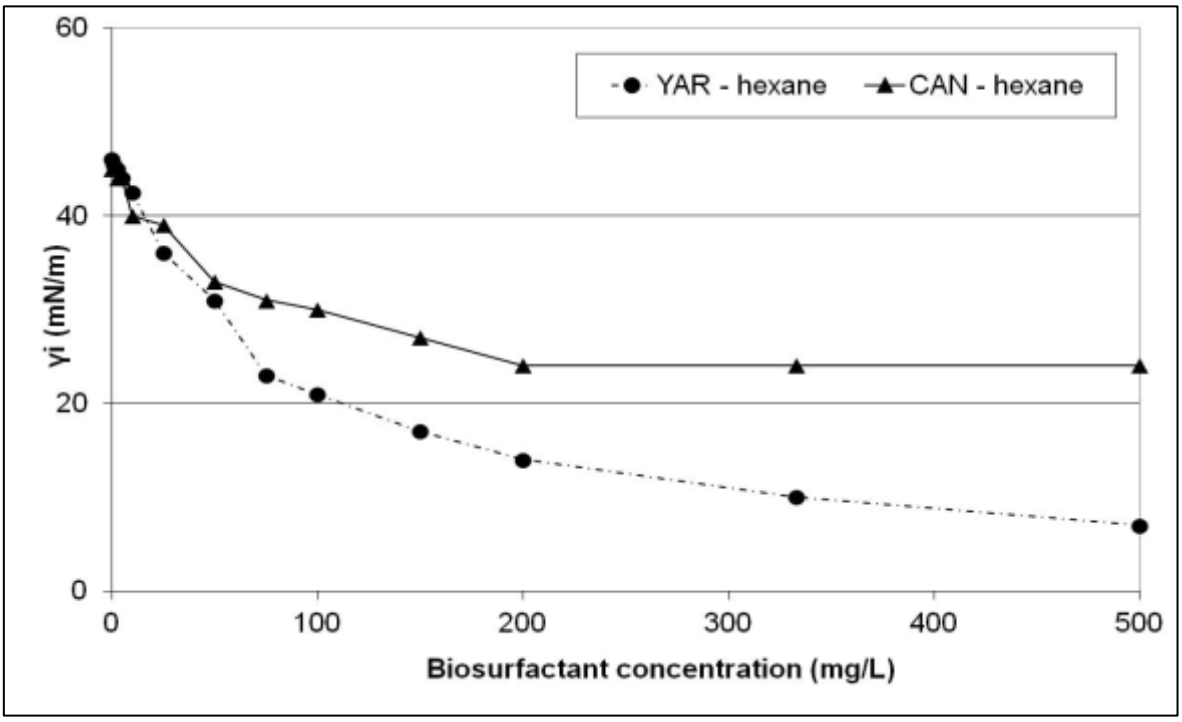


Fig. 7 Dependence of interface tension of the surfactant solution and hexane on YAR and CAN concentration (comparison).

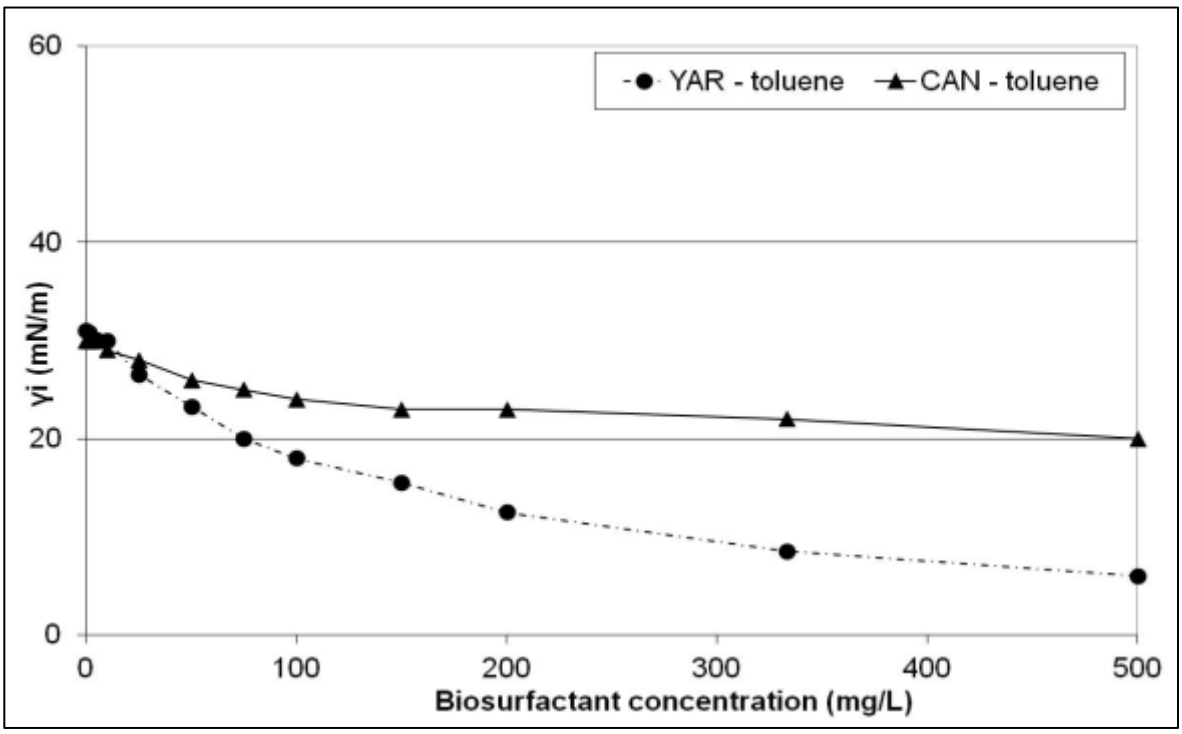


Fig. 8 Dependence of interface tension of the surfactant solution and toluene on YAR and CAN concentration (comparison).

Biosurfactant YAR was more efficient in decreasing the interface tension in the two-phase system with kerosene than biosurfactant CAN (Fig. 6). The addition of the biosurfactant YAR into solution decreased interfacial tension up to 84% for the system with kerosene. Addition of the biosurfactant CAN decreased interfacial tension only to 28% with kerosene.

Higher efficiency of biosurfactant YAR than biosurfactant CAN in decreasing of the interface tension in the two-phase system was proved also in system with hexane. The interfacial tension was decreased up to 80% by the addition of YAR and 47% by the addition of CAN (Fig. 7).

Results shown in Fig. 8 demonstrate in general higher efficiency of biosurfactant YAR in decreasing of the interface tension in the two-phase system with kerosene, hexane and toluene than biosurfactant CAN. The addition of the biosurfactant YAR into solution decreased interfacial tension of the system with toluene up to 80%, while biosurfactant CAN decreased interfacial tension to 33%.

3.5 Solubilization of Toluene

Solubilization of toluene was tested with YAR solutions in concentration range 0-500 mg/L. Results are shown in Fig. 9.

The amount of toluene solubilized in biosurfactant grows significantly from 100 mg/L to 150 mg/L of YAR (Fig. 9), thus effective concentration is above CMC (CMC = 12 mg/L). Weight solubilization ratios in examined system are considerably high: milligram of toluene per milligram of biosurfactant (YAR).

Solubilization of HCH isomers was tested with YAR and CAN solutions in concentration range 0-500 mg/L. Technical HCH is a mixture of isomers alpha (65%-70%), gamma (12%-15%), beta (6%-8%), delta (2%-5%) and epsilon (5%-10%). The results shown in Figs. 10 and 11 present solubilization of isomers alpha and gamma, forming the highest proportion of HCH. Solubilization of the other isomers was insignificant.

Both biosurfactants YAR and CAN show good solubilizing properties with HCH isomers alpha and gamma. The effective concentration of both biosurfactants is approximately 100 mg/L (Fig. 10).

The weight solubilization ratio in the concentration range 70-500 mg/L equals 2-5 mg (HCH)/g (YAR) for isomer alpha and 6-9 mg (HCH)/g (YAR) for isomer gamma. The weight solubilization ratio in the concentration range 70-500 mg/L equals 2-4 mg (HCH)/g (CAN) for isomer alpha and 5-7 mg (HCH)/g (CAN) for isomer gamma. Both biosurfactants YAR and CAN can be used for solubilization of technical HCH.

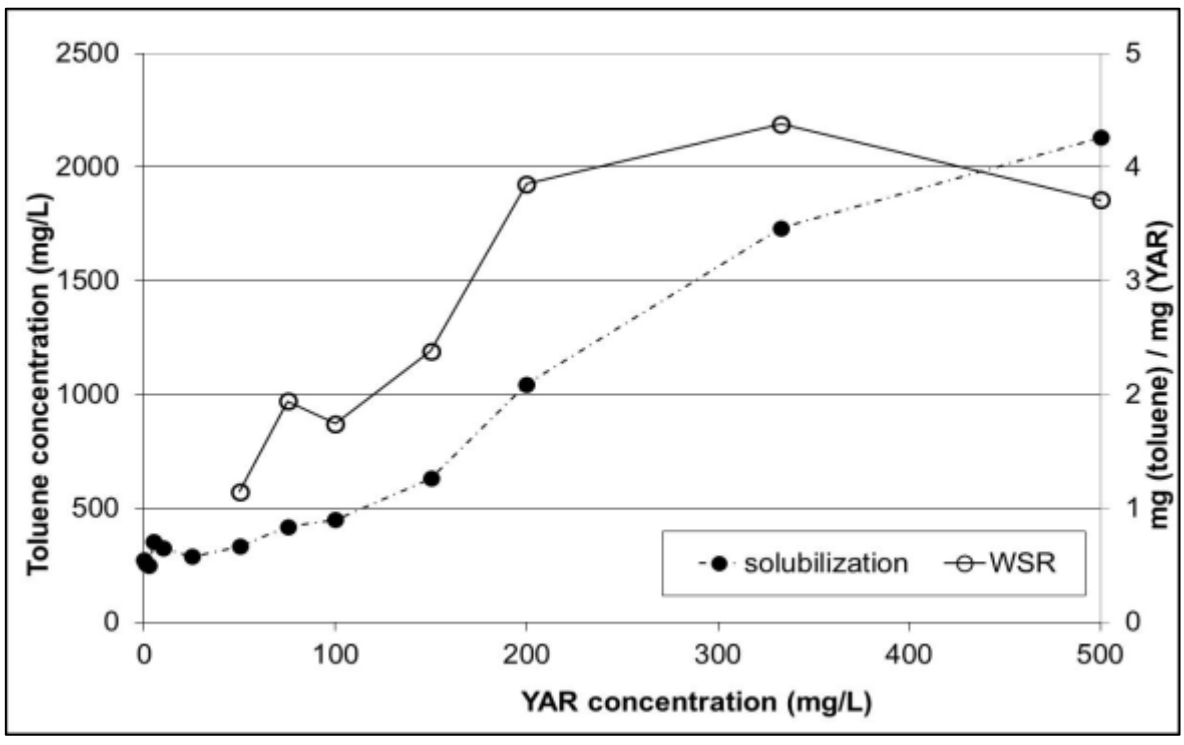


Fig. 9 Solubilization of toluene with biosurfactant YAR and weight solubilization ratio (WSR) of toluene and biosurfactant YAR.

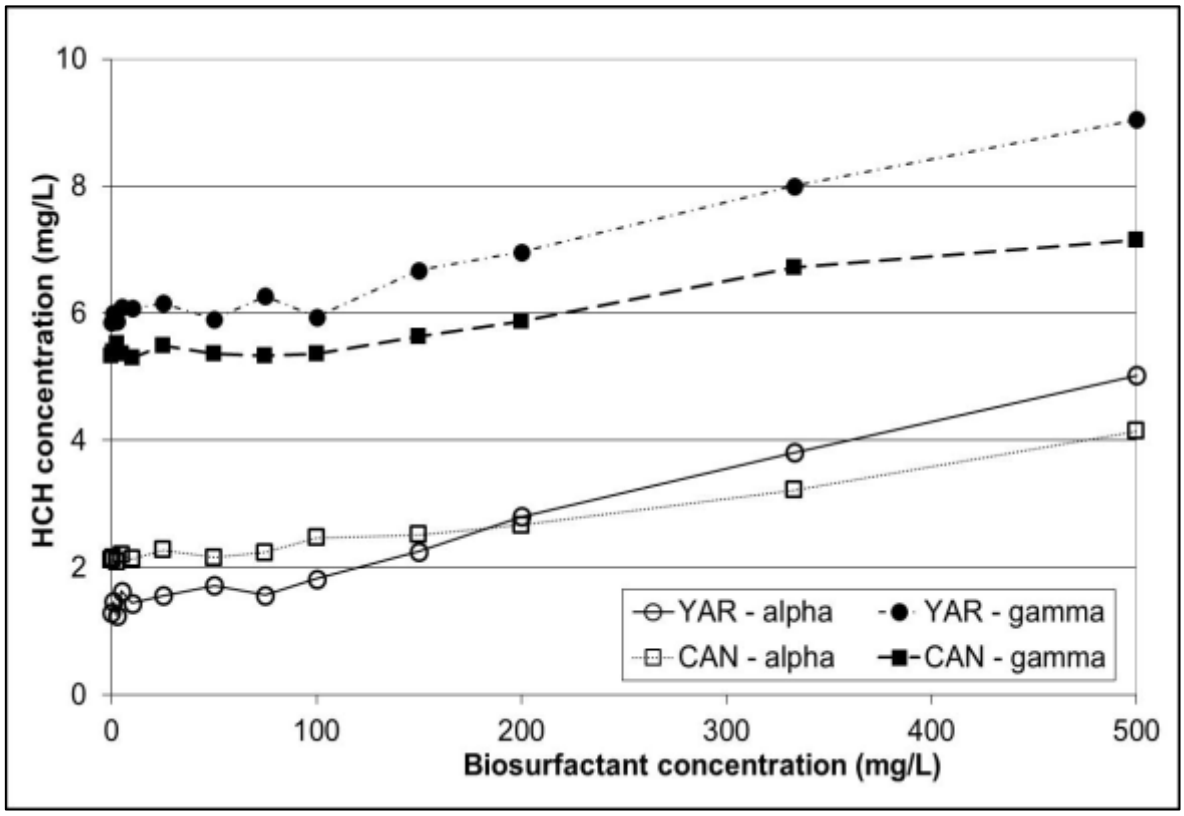


Fig. 10 Solubilization of HCH (isomers alpha and gamma) with biosurfactants YAR and CAN.

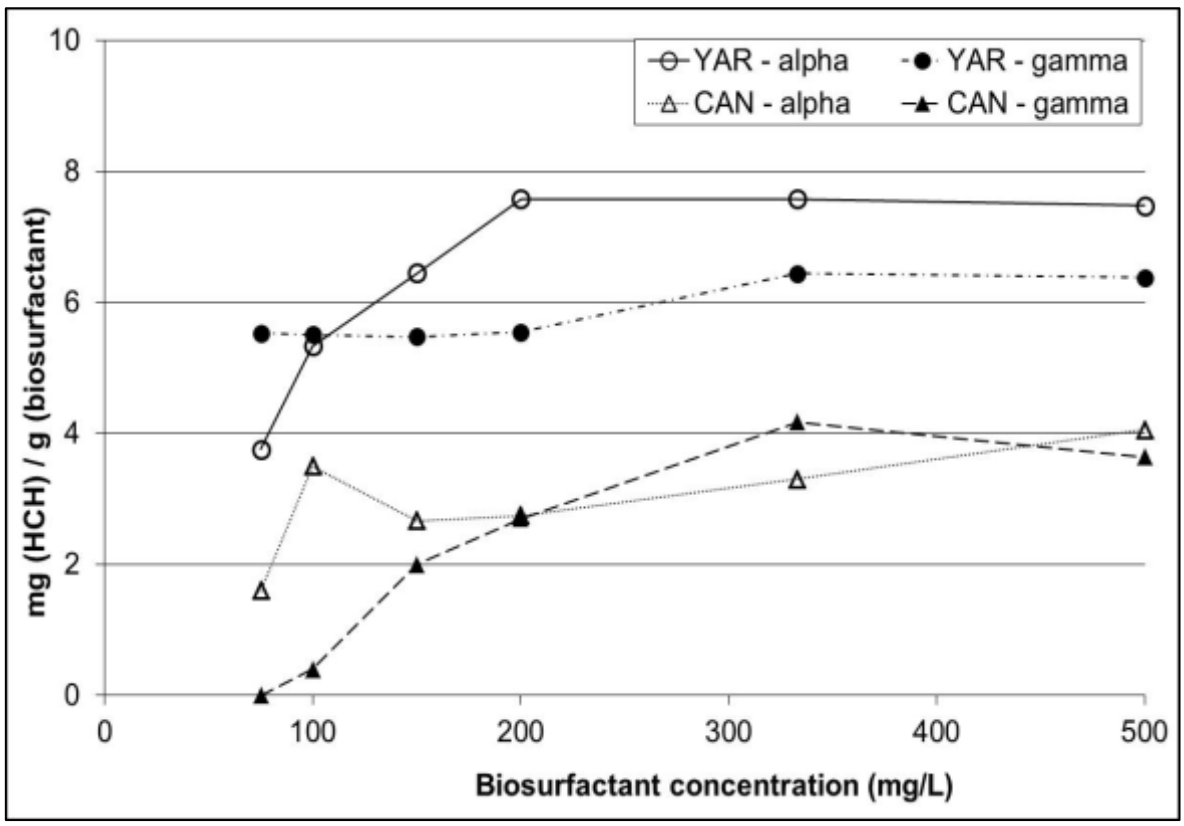


Fig. 11 Weight solubilization ratio of HCH (isomers alpha and gamma) and biosurfactants YAR and CAN.

The weight solubilization ratio of HCH was a bit higher, and thus more efficient for YAR than for CAN.

4. Conclusions

Basic properties of isolated biosurfactants depended mainly on the cultivation conditions and purity of the final product. Biosurfactants YAR and CAN were both able to reduce interfacial tension in all representative systems with model contaminants, and higher efficiency was observed for YAR (by 80%). Solubilization properties of YAR were proved with toluene and HCH isomers alpha and gamma. Both biosurfactants YAR and CAN showed good solubilizing properties with HCH isomers alpha and gamma.

Both isolated yeast biosurfactants studied in this work are promising agents in the removal of non-polar contaminants from the environment with several possibilities of their application. Both biosurfactants could be used in form of biosurfactant solution as a solubilization agent in soil washing and replace synthetic surfactants. The alternative is enhanced bioremediation, when biosurfactant causes contaminant release from the solid matrix, making it accessible to biodegradation by soil microorganisms. In order to reduce costs associated with isolation of the biosurfactant, infiltration of the culture medium with already produced biosurfactant and adapted yeast biomass to the soil could work as a connection of those two principles and the yeast can work as a bioremediation microorganism.

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References

[1] M. Pacwa-Plociniczak, G.A. Plaza, Z. Piotrowska-Seget, S.S. Cameotra, Environmental applications of biosurfactants: Recent advances, Int. J. Mol. Sci. 12 (2011) 633-654.

[2] I.N.A. Van Bogaert, Z. Jinxin, W. Soetaert, Microbial production of sophorolipids, Process Biochem. 46 (2011) 821-833.

[3] A.K. Mukherjee, K. Das, Microbial surfactants and their potential applications: An overwiew, in: R. Sen (Ed.), Biosurfactants, Springer, 2010, pp. 54-60.

[4] P.F.F. Amaral, J.M. Silva, M. Lehocky, A.M.V. Barros-Timmons, M.A.Z. Coelho, I.M. Marrucho, et al., Production and characterization of a bioemulsifier from *Yarrowia lipolytica*, Process Biochem. 41 (2006) 1894-1898.

[5] J.D. Desai, I.M. Banat, Microbial production of surfactants and their commercial potential, Microbiol. and Mol. Biol. Rev. 61 (1997) 47-64.

[6] J.A. Casas, F. García-Ochoa, Sophorolipid production by *Candida bombicola*: Medium composition and culture methods, J. Biosci. and Bioeng. 88 (1999) 488-494.

[7] J. Ruiz-Herrera, R. Sentandreu, Different effectors of dimorphism in *Yarrowia lipolytica*, Arch. Microbiol. 178 (2002) 477-483.

[8] M.A.Z. Coelho, P.F.F. Amaral, I. Belo, *Yarrowia lipolytica*: An industrial workhorse, in: A. Méndez-Vilas (Ed.), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex, Badajoz, 2010, pp. 930-940.

[9] S. Techaoei, P. Leelapornpisid, D. Santiarwarn, S. Lumyong, Preliminary screening of biosurfactant-producing microorganisms isolated from hot spring and ganges in Northern Thailand, Sci. Tech. J. 7 (2007) 38-43.