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Effect of exogenously added rhamnolipids on citric acid production yield

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The influence of a biosurfactant (rhamnolipids) on the effectiveness of citric acid production by *Yarrowia lipolytica* from sunflower oil was studied. The surfactant-mediated solubilization of the hydrophobic substrate was assessed by particle size distribution characteristics with and without the presence of sunflower oil hydrolization products. The presence of rhamnolipids contributed to a decrease of the oil droplet size, most notably for samples containing sunflower oil and its hydrolization products. The citric acid yield for cultures not supplemented with rhamnolipids was at 82.9 g/l, with a 1:0.04 citric acid to isocitric acid ratio (CA:ICA). The addition of rhamnolipids at 1 g/l resulted in a 5% increased citric acid yield (87.1 g/l), however a decrease (79.0 g/l) was observed for samples containing 5 g/l of rhamnolipids. The rhamnolipids-induced emulsification of sunflower oil did not seem to influence the citric acid production efficiency. Additional research revealed that the biosurfactant was degraded by yeast cells during the bioconversion process. The possible explanations of this phenomenon include the utilization of rhamnolipids as an alternative carbon source or microbial destabilization of micelles formed by this biosurfactant due to potential bioavailability issues.

Key words: *Yarrowia lipolytica*, citric acid, rhamnolipids, sunflower oil.

INTRODUCTION

The politics of EU countries regarding chemical technology and production strategies are influenced by the "green chemistry" concept, which is why an increasing need to explore the benefits of biotechnology to a greater extent could be observed over the last decade (Wache et al., 2003). As a result, an overproduction of vegetable oils occurred and this resource has been considered as a valuable substrate for bioconversion processes. The excessive oils were mostly used for the production of biodiesel, although, recently a lot of focus has been directed towards obtaining other highly desirable products such as citric acid (Kamzolova et al., 2005; Alam et al., 2008). The production of citric acid by biotechnological means is a well established and thoroughly researched process. Several reports confirm that yeast belonging to genera *Candida*, *Pichia*, *Torula*, *Rodotorula*, *Nocardia*, *Nematospora*, *Saccharomyces*, *Zygosaccharomyces* are capable of producing citric acid from various carbon sources (Finogenova et al., 2002; Crolla and Kennedy, 2004; Anastassiadis et al., 2005).

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However, it should be pointed out that achieving efficient bioconversion of vegetable oils into citric acid may be challenging, taking into account the presence of two immiscible phases. Poor aquatic solubility of the hydrophobic substrates contributes to limited bioavailability and is regarded as the main factor which inhibits the bioconversion rate (Angelova and Schmauder, 1999). This issue is of great importance in many different areas of biotechnology, where highly desirable products are formed by hydrophilic microorganisms from hydrophobic substrates (Carreira et al., 2001).

Since the uptake of water insoluble substrates is considered as a major limiting step, it has been established that increasing the contact area between the microorganisms and substrates may be of great biotechnological potential (Fickers et al., 2005). A common strategy applied in order to achieve a higher bioconversion yield of hydrophobic substrates involves the addition of surfactants (Singh and Singh, 2008). Surfactants are well known for their ability to accumulate at the oil-water interface and to increase the solubility of hydrophobic substances (Singh et al., 2007). The surfactant-mediated transport of water immiscible substrates into the aquatic phase may help to overcome the bioavailability limitations and improve the overall process efficiency. Additionally, some reports have shown the stimulatory effect of surfactants on the production of enzymes by microorganisms (Guerzoni et al., 2001). The potential enhancement was especially anticipated for surfactants of biological origin, since it was considered that the secretion of such compounds was associated with substrate uptake mechanisms (Boulton and Ratledge, 1984). Biosurfactants are a group of surface active compounds produced extracellularly or as a part of the membrane by various microorganisms for example, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Rhodococcus* species, *Candida bombicola*, *Streptomyces* and *Penicillum* species (Zinjarde and Pant, 2002). Because of their advantages over synthetic surfactants, they are often used in the cosmetic, pharmaceutical and food industry as well as bioremediation. Several biosurfactants exhibited low surface tension, low critical micelle concentration (CMC), high affinity for hydrophobic organic molecules and high emulsifying activity (Mulligan, 2005). They are also considered to be easily biodegradable, biocompatible and environmentally friendly (Mulligan, 2009).

Taking the aforementioned statements into account, we decided to study the effect of an environmental friendly biosurfactant, with a high average emulsifying activity and high affinity for hydrophobic molecules, on the effectiveness of hydrophobic substrate usage during citric acid production.

The objective of this research was to evaluate whether the exogenous addition of rhamnolipids will have a significant impact on citric and isocitric acid production by *Yarrowia lipolytica* yeast cells cultivated on sunflower oil. Changes in the particle size distribution profiles of sunflower oil upon rhamnolipids-supplementation were analyzed in order to examine the emulsification effect. Cell surface hydrophobicity changes were measured to assess the influence of rhamnolipids on yeast cells. Additionally, the concentration of rhamnolipids was monitored in order to check whether the biosurfactant was degraded during the bioconversion process.

MATERIALS AND METHODS

Chemicals

Sunflower oil (triglyceride composed of glycerin and linoleic, oleic, stearic and palmitic fatty acid moieties) was obtained from the commercial market (Kujawski, Poland). Rhamnolipids used throughout this research were produced at Poznan University of Technology by the bacterium *P. aeruginosa* TK (Chrzanowski et al., 2009), according to procedure described by Sim et al. (1997) and later purified as suggested by Arino et al. (1996). The composition of obtained rhamnolipids mixture was analogous to commercial JBR-425 produced by the Jeneil Biosurfactant Company, Saukville, WI, USA.

Microorganisms

Y. lipolytica H181 used throughout this study belongs to the UFZ Helmholtz Centre for Environmental Research collection, Leipzig, Germany. This mutant strain is able to produce citric acid with a high selectivity (93 to 96%) from vegetable oils (Aurich et al., 2003; Förster et al., 2007).

Cultivation conditions

The experiments were carried out in 500 ml Erlenmeyer flasks. The flask cultures containing 100 ml of medium were inoculated with approximately 10^8 yeast cells. The cultures were grown aerobically at 30°C on a rotary shaker (130 rpm), in a medium optimized for citric acid production, according to Behrens et al. (1978) and Moeller et al. (2007). The basal medium was supplemented by refined, commercially available sunflower oil in the concentration of 70 g/l. Rhamnolipids were added to the medium in the concentration of 1, 2.5 and 5 g/l. The final pH of the medium was adjusted to 6.0. The cultures were with rhamnolipids and no oil was used as a control sample. The experiment was finished when no further increase in the citric acid content was observed.

Hydrolysation of sunflower oil

Sunflower oil hydrolysis products were prepared in order to evaluate their role during the emulsification process: 10 ml of sunflower oil were heated with 25 ml of 2M NaOH for 2 h. The obtained hydrolysis products were blended with sunflower oil to get a final concentration of 10, 25 and 50% (v/v).

Particle size distribution analysis

Changes of the sunflower oil particles' size distribution after biosurfactant and/or hydrolysis products supplementation were evaluated with Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK). Experimental conditions, in terms of composition, corresponded to those of the cultivation experiments, but in the absence of the yeast cells. Samples (100 ml) were mechanically shaken for 24 h. The aliquots of the samples (the whole cultivation broth) were

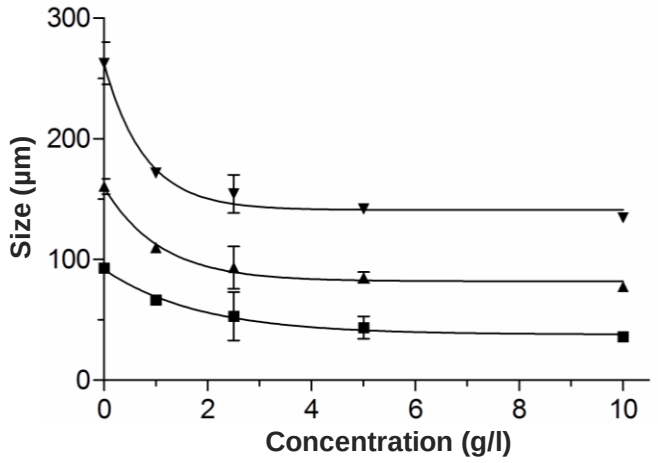

Figure 1. The effect of rhamnolipids concentration on the selected parameters of particle size distribution: ■ ,d(0.1); ▲ ,d(0.5); ▼ ,d(0.9).

subjected to the determination procedure following the manufacturer's instruction. Measurements were performed at 30°C. Three replicate experiments were carried out for each of the investigated conditions using the laser diffraction method realized in two stages: (i) sample is mixed with paddle agitator, then supplied to the optic unit, (ii) after completion of measurements and data collection, the Mastersizer software transforms the information into values, making use of the Mie theory and assuming the Fraunhofer model. The software uses a reflective index (RI) of 1.40 and a dispersant RI of 1.33 to calculate the dispersion index (Span) by the following equation:

$$\text{Span} = \frac{d(0.9) - d(0.1)}{d(0.5)}$$

Where, the d(0.1), d(0.5) and d(0.9) values are size values corresponding to cumulative distribution at 10, 50 and 90%, respectively.

Surface tension analysis

The ability of the microbial consortium to produce biosurfactants under the experimental conditions was investigated by employing the du Nouy ring technique described by Górna et al. (2011).

Cell surface hydrophobicity measurement

Changes in the cell surface hydrophobocity value were analyzed based on the MATH carried out as previously described in Owsianiak et al. (2009).

Citric and isocitric acid analysis

Organic acids were determined with an ion chromatographic system DX 600 (Dionex, Sunnyvale, USA) equipped with EG 40 KOH eluent generator, GS 50 gradient pump and CD 25 conductivity detector. For organic anion separation, an IonPac AS 11 (4 × 250 mm) analytical column (Dionex, USA) was used. Citric and isocitric acid were quantified using calibration curves with the software PeakNet 6.3 (Dionex).

Determination of rhamnolipids

The whole cultivation broth was subjected to extraction with hexane (3 × 20 ml, Julabo SW22 rotary shaker at 150 rpm for 10 min) three times in order to extract the residual sunflower oil. The hexane phase was rinsed three times with redistilled water (2 × 20 ml) and the aliquots were combined with the aqueous phase. In the next step, 50 ml of the aqueous phase were subjected to centrifugation at 10,000× *g* for 10 min in order to remove the residual biomass. Afterwards, the clear supernatant containing rhamnolipids was filtered through 0.22 µm Millipore and 10 ml was lyophilized in vials. The dry matter was derivatized according to the method described by Schenk et al. (1995), by dissolving in 1 ml of dry acetonitrile, which contained p-bromoacetophenone and triethylamine in a molar ratio of 1:4:2 (glycolipids:p-bromoacetophenone:Et3N). The derivatization reaction was carried out at room temperature for 18 h in a tightly closed vial with nitrogen as an inert gas. The obtained p-bromophenacyl rhamnolipid esters were then filtered (0.22 µm) and analyzed directly by HPLC.

The analysis was carried out using Aglient 1100 liquid chromatograph equipped with an UV-Vis detector and an Eclipse Plus C18 4.6 × 75 mm; 3.5 µm column. Water (solvent A) and acetonitrile (solvent B) was used as the mobile phase: 1 min, A:B, 30:70 (%), 5 min gradient from 30:70 to 0:100 (%), on hold for 5 min, balancing of the column for 3 min to 30:70. The analysis was carried out at 25°C with a flow rate of 2 ml per min at 265 and 320 nm.

Statistical analysis

A one-way ANOVA with Tukey's Multiple Comparisons statistical analysis was applied on the triplicate data of particle size distribution at alpha = 0.05 significance level using Statistica 6 PL (Statsoft Inc., Tulsa, USA) statistical software package.

RESULTS

Influence of rhamnolipids on emulsification of sunflower oil

The results obtained during the aggregate size distribution analysis indicate that rhamnolipids interacted with oil droplets, decreasing their diameter size (Figure 1). The most notable decrease occurred when rhamnolipids were applied at a low concentration, below 2.5 g/l with an average decrease of the diameter by 35%. This correlation can be observed in the d(0.5) µm column (Table 1), as those results are of greatest statistical significance. Further increase in the biosurfactant concentration had no effect on the particle size. The hydrolyzation of sunflower oil contributes to the release of saturated and unsaturated fatty acids, which exhibit surface activity due to their chemical composition. In order to evaluate whether the free fatty acids originating from the hydrolyzation process had any influence on the emulsification process, a separate set of studies was conducted in the presence and absence of rhamnolipids. The studies carried out for samples without rhamnolipids showed that blending sunflower oil with the hydrolysis products caused no statistically significant changes in the aggregate size distribution (Table

Table 1. Results of particle size distribution analysis after supplementation with rhamnolipids.

Rhamnolipids concentration (%)	d(0.1) (m)		d(0.5) (m)		d(0.9) (m)		Span	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	92.79	0.26	160.51	6.33	262.62	17.48	1.06	0.07
0.01	66.63	0.70	109.98	0.80	171.66	1.39	0.95	0.01
0.25	53.01	20.06	93.47	17.47	154.37	15.73	1.11	0.25
0.5	43.58	9.29	85.04	4.48	141.94	1.44	1.16	0.19
1	36.20	2.98	77.59	0.66	134.54	0.29	1.27	0.05

Table 2. Results of particle size distribution analysis after supplementation with sunflower oil hydrolysis products.

Hydrolysate concentration (%)	d(0.1) (m)		d(0.5) (m)		d(0.9) (m)		Span	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	92.79	0.26	160.51	6.33	262.62	17.48	1.06	0.07
10	84.49	16.39	157.95	12.61	269.46	4.55	1.75	0.16
25	60.08	7.97	149.39	6.43	274.04	8.85	1.06	0.12
50	58.27	9.53	162.21	9.54	318.19	13.23	1.60	0.22

Table 3. Results of particle size distribution analysis after supplementation with the mixture containing rhamnolipids (R) and sunflower oil hydrolysis products (HP).

Mixture composition		d(0.1) (m)		d(0.5) (m)		d(0.9) (m)		Span	
R (g/l)	HP (%)	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0	0	92.79	0.26	160.51	6.33	262.62	17.48	1.06	0.07
1.0	10	38.37	3.75	78.48	8.34	132.99	9.43	1.21	0.12
2.5	25	10.04	2.33	61.10	6.44	113.84	10.23	1.61	0.09
5.0	50	2.44	0.07	59.25	2.32	111.10	2.91	1.84	0.01

2). These results suggest that sole sunflower oil hydrolysis products do not actively take part in the emulsification process. On the other hand, interesting results were observed when both rhamnolipids and sunflower oil hydrolysis products were applied. The combination proved to be most efficient at decreasing the droplet diameters (Table 3). In this case, the aggregate size was progressively reduced with increasing concentration of both rhamnolipids and sunflower oil hydrolysis products, reaching approximately 65% final reduction of the d(0.5) µm value.

Influence of rhamnolipids on cell surface properties of *Yarrowia lipolytica* H181

The results of surface tension analysis revealed that no statistically significant changes could be observed for supernatant samples from cultivation on sunflower oil (data not shown). Since the decrease of surface tension values was marginal, it was established that *Y. lipolytica* H181 was unable to produce any bioemulsifier under the studied cultivation conditions. It was concluded that the introduction of an exogenous biosurfactant, such as rhamnolipids, may facilitate the contact between yeast cells and the hydrophobic substrate and contribute to an enhanced citric acid production efficiency. The analysis of cell surface hydrophobicity changes carried out according to the MATH assay revealed that *Y. lipolytica* H181 exhibited high hydrophobicity (approximately 65%) upon cultivation on sole sunflower oil.

The addition of rhamnolipids to cultures cultivated on sunflower oil caused a notable decrease of cell surface hydrophobicity at all the studied concentrations (21, 17 and 12% for cultures supplemented with 1, 2.5 and 5 g/l of rhamnolipids, accordingly). The lowest cell surface hydrophobicity (below 10%) was observed for cells cultivated solely in the presence of rhamnolipids.

Influence of rhamnolipids on citric acid production yield

During cultivation without supplementation with rhamnolipids, citric acid was produced at a maximum amount of 82.9 g/l, while isocitric acid was produced at 3.4 g/l, respectively. The volumetric rate of citric acid formation was Q_{CA} = 0.35 g/(l*h), the substrate conversion yield $Y_{CA/S}$ was 1.18 g/g and CA:ICA ratio was 1:0.04. When rhamnolipids were introduced into cultures cultivated on

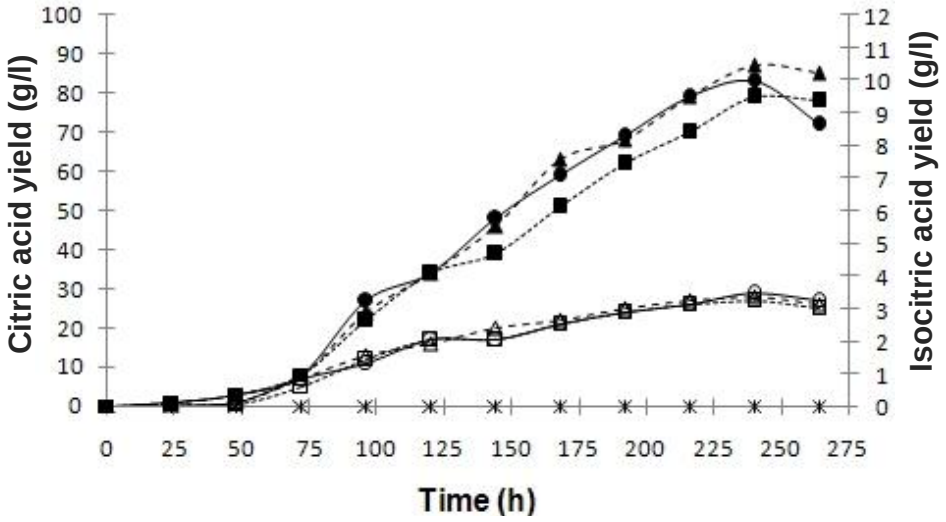

Figure 2. Course of citric and isocitric acid formation by *Y. lipolytica* H181 from sunflower oil with and without the addition of rhamnolipids: ●, citric acid without rhamnolipids; ▲, citric acid supplemented with 1 g/l of rhamnolipids; ■, citric acid supplemented with 5 g/l of rhamnolipids; ○, *iso*citric acid without rhamnolipids; Δ, *iso*citric acid supplemented with 1 g/l of rhamnolipids; □, *iso*citric acid supplemented with 5 g/l of rhamnolipids.

Table 4. Kinetic data for citric acid production by *Y. lipolytica* H181 with exogenously added rhamnolipids.

Rhamnolipids concentration (g/l)	Citric acid (g/l)	Isocitric acid (g/l)	Total yield (g/l)	CA:ICA ratio	$Y_{CA/S}$ (g/g)	Q_{CA} (g/l*h)	$Q_{CA, max}$ (g/l*h)	$q_{CA, max}$ (h^{-1})
0	82.9	3.4	86.3	1:0.04	1.18	0.35	0.53	0.11
1.0	87.1	3.5	90.6	1:0.04	1.24	0.36	0.55	0.12
5.0	79.0	3.7	82.7	1:0.05	1.13	0.33	0.52	0.10

CA , citric acid; ICA , isocitric acid; $Y_{CA/S}$,mass yield of citric acid; Q_{CA} ,volumetric rate of citric acid formation; $Q_{CA, max}$,maximal volumetric rate of citric acid formation; $q_{CA, max}$,maximal specific rate of citric acid formation.

sunflower oil, a slight impact on the citric acid production efficiency could be observed, depending on the concentration of the biosurfactant (Figure 2). The addition of rhamnolipids at a concentration of 1 g/l contributed to a 5% increase of citric acid production efficiency, reaching a maximal yield of 87.1 g/l. The volumetric product formation rate and substrate conversion yield reached R_{CA} = 0.36 g/l and $Y_{CA/S}$ = 1.24 g/g, accordingly. On the other hand, supplementation of rhamnolipids at a concentration of 5 g/l caused a 5% decrease of citric acid yield (79 g/l). The volumetric product formation rate and substrate conversion yield were lowered to R_{CA} = 0.33 g/l and $Y_{CA/S}$ = 1.13 g/g, accordingly.

Additionally, no significant changes in the CA to ICA ratio occurred upon the addition of rhamnolipids regardless of the concentration used. However, a slight increase in the isocitric acid yield was observed with increasing concentration of rhamnolipids (Table 4).

Biodegradation of rhamnolipids

The analysis of changes in the concentration of rhamnolipids during the cultivation of *Y. lipolytica* H181 on sunflower oil revealed that the biosurfactant molecules were depleted during the citric acid production process (Figure 3). The concentration of rhamnolipids started to decrease after a 3-day long lag phase. The decrease was more apparent for cultures cultivated in the presence of 1 g/l of rhamnolipids, with a 32% reduction of the initial amount (final concentration at approximately 780 mg/l). For cultures supplemented with 5 g/l of rhamnolipids, a 7% reduction of the initial amount was observed (final concentration at approximately 4550 mg/l). It is worth noticing that no statistically significant changes in the concentration of rhamnolipids occurred for blanks (samples without yeast). A separate set of studies, where rhamnolipids were used as a sole carbon and energy source (cultivation without sunflower oil) confirmed that the yeast cells were able to utilize the biosurfactant (up to 200 mg/l per day), although, no notable production of citric acid was observed.

DISCUSSION

Due to poor water solubility, the bioconversion of hydrophobic substrates is directly inhibited by mass transfer

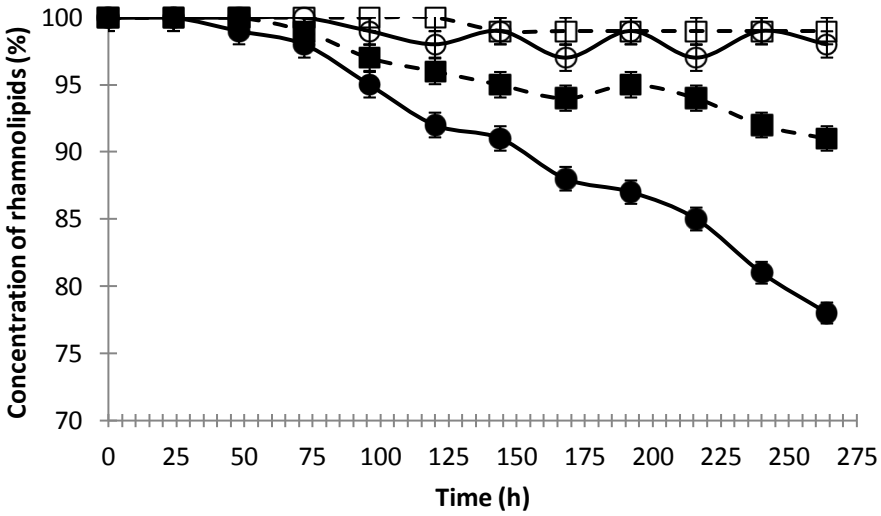

Figure 3. Changes in the concentration of rhamnolipids during the cultivation of *Y. lipolytica* H181: ●, cultures supplemented with 1 g/l of rhamnolipids; ■, cultures supplemented with 5 g/l of rhamnolipids; ○, blank (no yeast) supplemented with 1 g/l of rhamnolipids; □, blank (no yeast) supplemented with 5 g/l of rhamnolipids.

limitations. Taking this into consideration, it was initially expected that the utilization of sunflower oil as a carbon source may require certain microbial adaptations (such as secretion of a bioemulsifier) in order to enhance direct incorporation of the substrate into the membranes and cellular energy reserves (single cell oil) (Papanikolaou et al., 2002). Several reports confirmed that *Y. lipolytica* is able to produce a wide spectrum of products, some of which possess surface-active properties (Cirigliano et al., 1984; Amaral et al., 2006). Additionally, as shown in our previous studies, the substrate used for cultivation may have a fundamental influence on the composition of intracellular fatty acids as well as those found in the bioemulsifier produced by *Y. lipolytica* cells (Szulc et al., 2009). However, the *Y. lipolytica* H181 strain employed in this study did not exhibit the ability to secrete a bioemulsifier under the studied experimental conditions. Due to this reason, the effect of an exogenously added biosurfactants, rhamnolipids, was evaluated with the intent to enhance the citric acid production rate. It is commonly considered that biosurfactants-induced facilitation of the bioconversion rate may occur via two hypothetical transport mechanisms for hydrophobic substrates: surfactant-mediated solubilisation or enhancement of direct uptake through membrane modification, both of which have been previously reported for *Y. lipolytica* (Kim et al., 2000; Chrzanowski et al., 2008).

Subsequent investigation with the use of the aggregate size distribution analysis confirmed the occurrence of the first mechanism during this study. The decrease of the average droplet size in the presence of the rhamnolipids suggests that sunflower oil particles were successfully solubilized and dispersed in the aquatic phase. This effect was clearly visible, since the addition of rhamnolipids led to the formation of a stable milky emulsion. It is also worth noticing that the emulsifying effect of rhamnolipids was enhanced by the presence of sunflower oil hydrolysis products. A hypothetical explanation of this effect includes the occurrence of synergic interactions between both compounds during solubilization of oil droplets and formation of micelles which resulted in a more stable emulsification process. During the experiments, it was also observed that the biosurfactants molecules interacted with yeast cells by modifying their surface characteristics. However, since the addition of rhamnolipids resulted in decreased cell surface hydrophobicity, no possible enhancement via direct uptake was possible. The decrease of yeast cell hydrophobicity upon introduction of rhamnolipids has been previously reported (Chrzanowski et al., 2008; Kaczorek et al., 2008). Al-Tahhan et al. (2000) has established that this phenolmenon is associated with rhamnolipids-induced removal of lipopolysaccharides (LPS) from cell membranes.

Although, solubilization and emulsification of the hydrophobic substrate was achieved, it did not contribute to significant differences in citric acid yield. The citric and isocitric acid production rates during the cultivation with and without rhamnolipids were very similar. It should be pointed out that the *Y. lipolytica* H181 strain used throughout this study was capable of effectively utilizing sunflower oil for the production of citric acid even without the addition of biosurfactants. The obtained results were comparable with those reported by Rywińska et al. (2010), thus proving that the employed strain is an efficient citric acid producer. Overall, since only a slight increase observed for biosurfactants-mediated samples, it was established that this strategy is not valid. In order to elucidate the reason why no enhancement of citric acid production rate

was observed despite the solubilization of sunflower oil, additional studies were carried out.

During these tests, it was observed that the concentration of rhamnolipids was progressively depleted during the cultivation due to their biodegradation by the yeast cells. This observation corresponds well with the findings of our previous study, where simultaneous biodegradation of rhamnolipids and diesel oil by a soil isolated microbial consortium was observed (Chrzanowski et al., 2012). During the previous research, it was observed that rhamnolipids were dissipated more rapidly than the hydrophobic diesel oil hydrocarbons. Since no notable effect on citric acid production efficiency in the presence of rhamnolipids was observed during this study, the occurrence of a co-metabolic effect is rather unlikely.

The biodegraded rhamnolipids were most likely used by the yeast for generation of biomass or transformed into other metabolites. Interestingly, although, rhamnolipids are commonly considered as easily biodegradable compounds (Mulligan, 2005), the number of studies related to this topic is limited. Reports that focus on utilization of rhamnolipids by yeast cells are even rarer. To our knowledge, this is the first time when biodegradation of this biosurfactants by *Yarrowia* cells was observed, thus making it a novel discovery.

We hypothesize that the simultaneous biodegradation of both sunflower oil and rhamnolipids occurred due to possible entrapment of oil droplets into biosurfactant micelles. Like all surface-active agents, rhamnolipids display a tendency to deposit at the oil-water interface. Their presence may potentially inhibit the activity of lipases, which are a group of enzymes responsible for the biological breakdown of sunflower oil. It cannot be excluded, that while rhamnolipids solubilized the oil droplets, at the same time, they may have limited the adsorption of lipases to the interfacial boundary and delay their activation or otherwise influence certain factors which are crucial in terms of lipase activity (that is, surface pressure) (Rao and Damodaran, 2002). Guha and Jaffe (1998) established that with increasing concentration of surfactants, the bioavailability of hydrophobic compounds in the aquatic phase is decreased. Several recent studies confirm that the bioavailability of hydrocarbons deposited inside the micelle core and at its surface may or be limited for microorganisms (Chrzanowski et al., 2009, 2011). Therefore, although, the introduction of rhamnolipids into the cultures increased the apparent solubility of sunflower oil droplets in the water phase, this achievement may have been negated by the fact that the emulsified substrate was not readily bioavailable for the microorganisms. Thus, it is plausible, that the microbial attack on micelles was carried out in order to improve the accessibility of the hydrophobic substrates. On the other hand, rhamnolipids may have simply served as an alternative carbon source.

Analyzing the lipase activity and bioavailability of substrates during rhamnolipids-supplemented production of citric acid from hydrophobic substrates may provide further insight into this phenomenon and will be the subject of future studies.

Conclusion

The results regarding the interactions between rhamnolipids, sunflower oil particles and yeast cells are not unequivocal. It was observed that the addition of rhamnolipids contributed to the solubilization of the hydrophobic substrate and formation of a stable emulsion. On the other hand, the biosurfactant interacted with yeast cells by notably decreasing their hydrophobicity, which may have resulted in the reduction of direct substrate uptake. It seems that both processes did not notably influence the citric acid production process, since only slight differences in the citric acid yield could be observed, regardless of biosurfactant concentration. Overall, it was established that the supplementation of rhamnolipids is a failure in terms of process economics, due to high costs and lack of sustainability. However, it is worth noticing that the biodegradation of rhamnolipids by the yeast cells was observed during additional studies. This discovery is very interesting and may potentially be crucial in explaining the mechanism of substrate uptake in an emulsified environment.

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