BIOSURFACTANTS – BIODEGRADABILITY, TOXICITY, EFFICIENCY IN COMPARISON WITH SYNTHETIC SURFACTANTS

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ABSTACT
Biosurfactants are natural surface active agents produced by variety of bacteria yeast and fungi. A review of their biodegradability, toxicity and efficiency is presented in order to compare their properties with synthetic surfactants. Due to their high efficiency in many applications and environmentally friendly character we tried to apply them in oil refining process. The efficiency of biosurfactants in this application was very high and reached up to 99%.

KEYWORDS
biosurfactants application, efficiency, toxicity, synthetic surfactants, vegetable oil refining, phosphorus removal

INTRODUCTION

Surface active compounds (SACs) are one of the most commonly used chemicals in everyday life. From the beginning of XX century the production of wide spectrum of synthetic surfactants from petroleum resources has risen intensively. But even long time before some natural surfactants, like soap (fatty acid salt), lecithin (phospholipid) or saponins (glycolipid), were widely used in households and industry (Kitamoto et al., 2002). The term *natural surfactants* refers to SACs of vegetable and animal origin, obtained by extraction, precipitation or distillation, and SACs of microbial origin (biosurfactants), obtained in fermentation processes (Paraszkiewicz and Dlugoński, 2003; Holmberg, 2001).

SACs in plants and animals kingdom are present in small amounts and the cost of their obtainment exceeds the cost of chemical synthesis. The competitive source of natural surfactants are microorganisms due to the economical aspect of natural surfactants production. Bacteria, yeast and fungi are effective producers of amphiphilic compounds and are in great interest of biotechnologists (Holmberg 2001). However, the lion part of so called *biosurfactants* is produced by bacteria and only few by yeast and fungi (Kitamoto et al. 2002).

Biosurfactants were first discovered as extracellular amphiphilic compounds of fermentation bacteria (Kitamoto et al. 2009). Initially they were seen interesting due to their ability to increase solubility of insoluble or poorly soluble hydrocarbons. However, the more and more popular trend of using renewable resources in industry (especially in food and farmaceutical industries) have led to relentless interesting in gaining and application of natural surfactants, mainly biosurfactants (Nitschke and Costa, 2007). Nowadays, biosurfactants are produced using co- and by-products of different technologies as a carbon source for microorganisms (molasses, glycerol, whey, frying oil, animal fat, soapstock and starch-rich wastes e.g. potato wastes) (Maneerat 2005; Makkar and Cameotra, 2002).

Biosurfactants are classified mainly on the basis of their chemical structure and origin. The hydrophilic head is usually aminoacid, peptide, mono-, di- or polysaccharide. The hydrophobic tail is usually saturated, unsaturated, linear, branched or hydroxylated fatty acid. In table 1 there are presented examples of five groups of biosurfactants with their microbial origin.

Table 1. Biosurfactants classification and microbial origin examples

Biosurfactant class		**Microbial strain**
Glycolipids	rhamnolipids	*Nocardioides* sp. (Vasileva-Tonkova and Geshevaa, 2005)
	sophorolipids	*Candida* sp. (Hirata et al., 2009)
	trehalose lipids	*Rhodococcus* sp. (Lang and Philip, 1998)
Lipopeptides and lipoproteins	fengycin	*Bacillus* sp. (Vanittanakom et al., 1986)
	arthrofactin	*Arthrobacter* sp. (Morikawa et al., 1993)
Phospholipids and fatty acids	bile salts	*Myroides* sp. (Maneerat et al., 2005)
	fatty acids	*Mycobacterium* sp., *Nocardia* sp., *Candida* sp., *Cladosporium* sp. (Rehm and Reiff, 1981)
	phosphatidylethanolamine	*Rhodococcus* sp. (Kretschmer et al., 1982)
Polymeric biosurfactants	alasan	*Acinetobacter* sp. (Navon-Venezia et al. 1995)
	bioemulsan BS29	*Gordonia* sp. (Franzettia et al., 2009)
Particulate biosurfactants	whole cells	*Yarrowia* sp. (Zinjarde and Pant, 2002)
	vesicles	*Serratia* sp. (Matsuyama et al., 1986)

BIOSURFACTANTS TOXICITY

There are little publications strictly devoted to toxicity of biosurfactants. Toxicity tests are rather a part of wider research over applicational functions. In spite of this biosurfactants are commonly considered as low- or non-toxic. Selected data on biosurfactants toxicity are presented in table 2.

As it can be seen from presented data, biosurfactants in comparison with synthetic surfactants pose haemolytic activity to human erythrocyte lower than cationic surfactants (CTAB, TTAB, BC) and anionic SDS. They do not pose detrimental effect to heart, lung, liver and kidney and interfere in blood coagulation in normal clotting time. Their inhibit luminescence of 50% of *Vibrius fisheri* in comparable or higher concentration than synthetic surfactants and affect mouse fibroblast viability in concentration 500 times higher than LAS. Moreover, their acute and chronic toxicity is much lower than that of synthetic Triton X-100.

BIOSURFACTANTS BIODEGRADABILITY

There are very little publications devoted at all to biodegradation of biosurfactants, however, basing on published data biosurfactants seem to be more easily biodegradable than their synthetic equivalents.

The biodegradability tests of sophorolipids biosurfactants produced by non-pathogenic yeast *Candida bombicola*, performed according to the OECD Guidelines for Testing of Chemicals (301C Modified MITI Test), showed that biodegradation of biosurfactants starts immediately after cultivation. Moreover, biodegradability, expressed in a form of BOD/TOD (Biochemical Oxygen Demand to Total Oxygen Demand ratio), for sophorolipids after 8 days of cultivation has reached the level of 61%. Two others biosurfactants (surfactin and arthrofactin) examined were also as easy biodegradable as sophorolipids, while synthetic surfactants showed no biodegradability after 8 days (Hirata et al., 2009).

Table 2. Comparison of toxicity and cytotoxicity of biosurfactants and synthetic surfactants

Toxicity test (test subject)	Biosurfactant (*microbial origin*) / surfactant	Toxicity, effect	Ref.
Hemolytic activity (human erythrocite, 37˚C)	Lipopeptide biosurfactant (*B. subtilis* ATCC 6633)	MHC 0.26 mg/ml (97%), cmc 0.25 mg/ml, (MHC/cmc = 1.04) — The lowest ability to rupture erythrocytes	Dehghan-Noudeh et al., 2005
	CTAB (hexadecyltrimethylammonium bromide)	MHC 0.15 mg/ml (99%), cmc 0.02 mg/ml, (MHC/cmc = 7.5) — The highest ability to rupture erythrocytes	
	TTAB (tetradecyltrimethylammonium bromide)	MHC 0.20 mg/ml (98%), cmc 0.035 mg/ml, (MHC/cmc = 5.7) — High ability to rupture erythrocytes	
	BC (benzalkonium chloride)	MHC 0.20 mg/ml (98.5%), cmc 0.035 mg/ml, (MHC/cmc = 5.7) — High ability to rupture erythrocytes	
	SDS (sodium dodecyl sulphate)	MHC 0.20 mg/ml (97%), cmc 0.05mg/ml, (MHC/cmc = 4) — High ability to rupture erythrocytes	
Antimicrobial activity (bacterial strains: *E. coli*, *K. cryocrescens;* yeast strains: *S. cerevisiae*, *D. hansenii*, *P. anomala*, *C. glabrata*, *R. solanacearum)*	Biosurfactant M* (*P. aeruginosa)*	Inhibition of microbial growth of all examined microorganisms except *Candida glabrata*	Das and Mukherjee, 2005
	Biosurfactant NM** (*P. aeruginosa)*	Inhibition of microbial growth of *Kluyver cryocrescens* only	
Hemolytic activity (human erythrocyte)	Biosurfactant M (*P. aeruginosa)**	Hemolysis 1.8±0.1 % — More potent in causing hemolysis	
	Biosurfactant NM (*P. aeruginosa)***	Hemolysis 1.3±0.1 % — Less potent in causing hemolysis	
In vitro tissue damage (heart, lung, liver, kidney)	Biosurfactant M (*P. aeruginosa)**	0 % hemoglobin release by 200 (µg/ml) of HPLC fraction — No detrimental effect	
	Biosurfactant NM (*P. aeruginosa)***		
Interference in blood coagulation (platelet-poor plasma from goat)	Biosurfactant M (*P. aeruginosa)**	Ca-clotting time 100.4±2 (s) — Interferation with the normal clotting time (beginning value 157±1 s)	
	Biosurfactant NM (*P. aeruginosa)***	Ca-clotting time 99.1±1 (s)	
Acute toxity (outbred male albino mice)	Glycolipid (*R. ruber* IEGM 231)	No effects on central nervous system, no deaths, weight losses and changes in behaviour	Kuyukina et al., 2007
Bioluminescence test (*V. fisheri*)	Glycolipid (*R. ruber* IEGM 231)	IC_{50} 650 ± 150 (mg/l)	Ivshina et al., 1998
	Trehalose dicorynomycolate (*R. erythropolis)*	IC_{50} 49 (mg/l)	
	Trehalose tetra ester (*R. erythropolis)*	IC_{50} 286 (mg/l)	
	Rhamnolipids (*P. aeruginosa)*	IC_{50} 50 (mg/l)	
	Nonylphenol-(ethylenoxide)9-acetate (EQ 9)	IC_{50} 78 (mg/l)	
	Sucrose stearate (DK 50)	IC_{50} 67 (mg/l)	
	Finasol OSR-5	IC_{50} 7 (mg/l)	
	Corexit 9597	IC_{50} 5 (mg/l)	
	Inipol EAP 22	IC_{50} 0.4 ± 0.2 (mg/l)	
Neutral Red assay (transformed mouse fibroblast L929 cells)	Mannosylerythritol lipid MEL (*Candida* sp. SY16)	48 NR_{50} 5 (g) — Not harmful to human skin and eyes	Kim et al., 2002
	LAS (linear alkylbenzene sulphonate	48 NR_{50} 0.01 (g)	
	SDS (sodium dodecyl sulphate)	48 NR_{50} 0.05 (g)	
Subacute toxicity (adult Sparague-Dawley rats, 28 day)	Surfactin C (*B. subtilis)*	No deaths of rats at any doses, highest doses significantly decreased body weight with normal food and water consumption	Hwang et al., 2009
Histhopathological tests (liver, lungs, heart, spleen, adrenals, kidneys, thyroid glands, testes, ovaries)		Significant increase in liver weigh with 1 and 2 (g/kg) doses (zonal necrosis of hepathic vein)	
Acute and chronic toxicity (estuarine epibenthic invertebrate *Mysidopsis bahia* and inland silverside *Mendidia beryllina)*	Bio-Em (*P. aeruginosa* SB 30)	LC_{50} (7d) >20.0 (*M. bahia*); 14.2 (*M. beryllina*) [mg/l] — FEC_s (7d) 16.8 (*M. bahia*); 15.5 (*M. beryllina*) [mg/l]	Edwards et al., 2003
	Emulsan (*A. alcoaceticus* RAG-1)	LC_{50} (7d) >200.0 (*M. bahia*); 300 (*M. beryllina*) [mg/l] — FEC_s (7d) - (*M. bahia*); 232.4 (*M. beryllina*) [mg/l]	
	PES-51 (mixture of D-limnoene and bacteria fermentation by-products)	LC_{50} (7d) 15.4 (*M. bahia*); 20.3 (*M. beryllina*) [mg/l] — FEC_s (7d) 10.1 (*M. bahia*); 21.7 (*M. beryllina*) [mg/l]	
	Corexit 9500 (blend of fatty esters, glycol ethers and oxyalkylates in a parafinic solvent)	LC_{50} (7d) >1000 (*M. bahia*); 408.0 (*M. beryllina*) [mg/l] — FEC_s (7d) - (*M. bahia*); 464.8 (*M. beryllina*) [mg/l]	
	PES-61 (mixture of orthosilicate polymer and similar bacterial fermentation by-products as PES-51)	LC_{50} (7d) 13.4 (*M. bahia*); 75.7 (*M. beryllina*) [mg/l] — FEC_s (7d) 4.2 (*M. bahia*); 77.5 (*M. beryllina*) [mg/l]	
	Triton X-100	LC_{50} (7d) 3.3 (*M. bahia*); 2.5 (*M. beryllina*) [mg/l] — FEC_s (7d) 2.8 (*M. bahia*); 2.3 (*M. beryllina*) [mg/l]	
Biolumiscence test (*V. fisheri)*	Pure rhamnolipid RL (*Pseudomonas* sp. PS-17)	EC_{50} 13 [mg/l] — Higher cytotoxicity against L929 and A549 cells (24, 48 and 72 h of contact)	Kolwzan et al., 2008
Cytotoxicity (mouse fibroblast L929cells and human lung cancer A549 cells)	Rhamnolipid biocomplex (BX) with alginate (not deeply purified RL) (*Pseudomonas* sp. PS-17)	EC_{50} 110 [mg/l] — Lower cytotoxicity against L929 and A549 cells (24, 48 and 72 h of contact)	

cmc – critical micellar concentration; MHC – maximal hemolytic concentration; FEC_s – first effect of survival concentration; NR_{50} – concentration of the test agent that reduced the uptake of NR (Neutral Red dye) by 50% as compared to untreated control cells; IC_{50} – inhibition concentration observed for half of population; EC_{50} – effective concentration observed for half of population; M* mucoid strain; NM** non-mucoid strain

Another research indicated that rhamnolipid biosurfactants are biodegradable under aerobic and anaerobic conditions (soluble COD removal efficiency of 74% after 10 d and 47.2% after 6 d, respectively), whereas synthetic surfactant Triton X-100 is non-biodegradable under anaerobic conditions and only partially biodegradable under aerobic conditions (soluble COD removal efficiency of 47.1% after 10 days at concentrations below 900 mg/L) (Mohan et al., 2006).

Mannosylerythritol lipid biosurfactant (MEL) produced by *Candida Antarctica* was easily biodegradated by activated sludge microorganisms (almost all biosurfactant degradated in 5 days), while LAS and SDS were hardly degradated after 7 days of incubation (LAS 75% and SDS 60% of relative amount of dissolved oxygen) (Kim et al., 2002).

An exopolysaccharide biosurfactant (EPS_{2003}) turned out to be easily biodegradable in marine environment by chosen bacterial strains and its mineralization in the case of *Pseudoalteromonas* sp. (isoDE-01 strain) exceeded 90%, whereas in the case of *V. proteolyticus* (isoDE-07 strain) mineralization was less effective, reaching only 60% (Cappello et al., 2011).

Biodegradability tests in liquid medium and in soil microsms, performed for five biological surfactants (produced by two *Bacillus* sp., *Flavobacterium* sp., *Dietzia maris* and *Arthrobacter oxydans*) and synthetic SDS, pointed that efficiency of their degradation depends on used bacteria. However, the biodegradability of all biosurfactants by mixed culture in soil did not differ significantly and ranged from 42,5% up to 73,4%, while biodegradability of synthetic SDS during 7 days of incubation was much lower (24.8%) (Lima et al., 2011).

Biodegradation of rhamnolipid in two types of soil (loamy and sandy soil) was relatively low in the first two days of incubation, but sharply increased on the third day and after seven days of incubation 92% of rhamnolipid was degraded in both kinds of soil examined (Pei et al., 2009). In another research it was completely degradated after 4 days of cultivation by bacterial mixed population isolated from soil (Fiebig et al., 1997).

BIOSURFACTANTS PROPERTIES AND APPLICATIONS

Physicochemical properties

Some investigations showed, that surface activity of biosurfactants is comparable with surface activity of synthetic surfactants. For example biosurfactants are able to reduce surface tension of water to 29.0 mN/m (at the cmc), while Pluronic F-68 to 42.8, SDS to 28.6 (Pornsunthorntaweea et al., 2008) and LAS to 31 mN/m (Kim et al., 2002). Moreover, water-in-oil emulsions of palm, crude, soybean, coconut and olive oils with biosurfactants were comparably or even more stable than that with synthetic surfactants. On the other hand, if oil phase are short-chain hydrocarbons, the emulsions are less stable with biosurfactants (Pornsunthorntaweea et al., 2008).

Biosurfactants are characterized by smaller than for synthetic surfactants critical micellar concentration (cmc) (0.07 and 0.12 g/l for biosurfactant rhamnolipid and Rokanol NL6, respectively) (Medrzycka et al., 2009). Therefore, in spite of a little smaller solubilisation efficiency (presented in a form of weight solubilisation ratio: 0.218 for biosurfactant and 0.277 for Rokanol NL6, (Pastewski et al., 2008)), they are more efficient in washing out oil from the ground. The maximal oil removal for biosurfactant solution was about 22%, while for synthetic surfactant 14% (Medrzycka et al., 2009).

Environmental applications

Due to good physicochemical properties, low toxicity and good biodegradability biosurfactants are widely applied in environmental protection techniques, *e.g.* water and soil remediation, oil spills removal etc.

Biosurfactants turned out to remove crude and model oils from sand columns or contaminated ground in the washing process. The efficiency of biosurfactant in removing crude oil was comparable to those of synthetic surfactant and much higher than for natural plant surfactant – saponin (Urum et al., 2006) and synthetic Tween 60 (Kuyukina et al., 2005). In the case of removing hexadecane from sand biosurfactant was much more efficient than SDS and Tween 80 (Bai et al., 1997).

Biosurfactants are also very effective in enhancing of oils biodegradation. The addition of biosurfactant produced by *Candida Antarctica* to the fermentation process of n-undecane improved degradation rate of petroleum hydrocarbons, while application of synthetic surfactant Tween 40 and Span 80 didn't show any improvement (Hua et al., 2003).

In biodegradation of phenanthrene biosurfactants produced by *Pseudomonas aeruginosa* P-CG3 and ATCC 9027 strains were less effective than synthetic Tween 80 and more effective than Triton X-100 in respect to enhancement of the rate of biodegradation. However, they displayed the highest phenanthrene solubilisation among all examined surfactants. The solubility of phenanthrene in P-CG3 and ATCC 9027 biosurfactants' solutions at 10 x CMC concentrations were 50 and 28 mg/l, respectively, while only 16 and 11 mg/l for Tween 80 and Triton X-100, respectively (Wong et al., 2003).

A review of investigations on efficiency of biosurfactants and synthetic surfactants in enhancing biodegradation of polycyclic aromatic compounds (PAHs) showed, that biosurfactants display similar PAH bioavailability enhancement as synthetic surfactant, however, are non-toxic to microorganisms degradating pollutants (Makkar and Rockne, 2003).

Summarizing, one can state that biosurfactants display a lot of advantages over chemically synthesized surfactants. They are less toxic, highly effective and easily biodegradable, what makes them environmentally friendly and proves their potential to replace synthetic surfactants in many applications, not only of environmental character. One of the possibility is exploiting their solubilising properties and using in vegetable oils refining for removal of phospholipids.

EXPERIMENTAL

In our research the rapeseed oil was experimentally refined (degumming process) with using biosurfactants solutions. During so called "degumming" process there are removed from oil substances of gummy character, mostly phospholipids. Phospholipids should be definitely removed from oil. If not, they undergo spontaneous hydration during storage, precipitate in oil in a form of sludge and negatively influence next refining steps. In classical technologies phospholipids are removed by hydration (removes hydratable phospholipids only, HP) or hydration with acid pretreatment (removes both, HP and non-hydratable phospholipids, NHP, but all of them are carbonised and their recovery is not possible). In the method presented here phospholipids are not destroyed and it is possible to recover and to use them. During the washing of oil with biosurfactant solution, phospholipids are removed thanks to formation of aggregates with biosurfactants molecules. The method of degumming of oil with using of biosurfactants was patented by Ukrainian scientists (Ukrainian patent 42406) however, the detailed parameters and mechanisms are not described.

MATERIALS AND METHODS

Not deeply purified biosurfactant produced by *Pseudomonas* sp. PS-17 was used as degumming (phospholipid removing) agent. It was obtained in Lviv Academy of Sciences (Ukraine). The preparation is a biocomplex of rhamnolipid and alginate, obtained by acidification of culture broth from *Pseudomonas* sp. PS-17 cultivation to pH 2 (Ukrainian patent 10467 A). Degumming process

was performed in such a way, that enriched in lecithin model oil (phosphorus content 300 mg/kg) was washed with biosurfactant solutions at room temperature. The changed parameters were concentration and pH of biosurfactant solutions and mass ratio of solution to oil.

RESULTS AND CONCLUSIONS

The efficiency of phospholipids removal from oil is presented in Figure 1 as phosphorus removal dependence (%) on X ratio, where X is mass of biosurfactants solution to mass of oil ratio in [g/g].

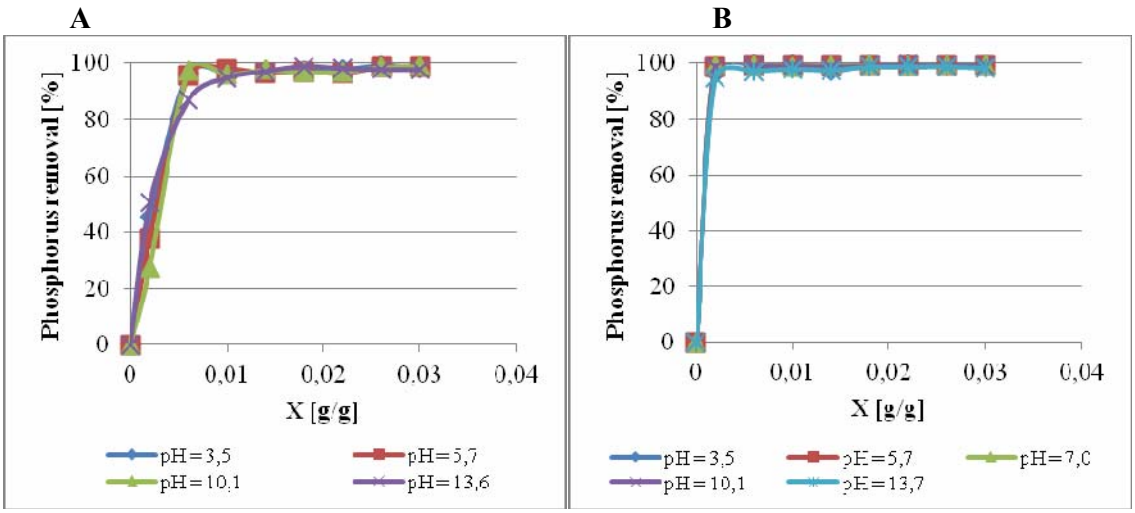

Figure 1. Phosphorus removal rate dependence on the amount of biosurfactants solution at 5,0 (A) and 2,5 g/l (B) concentration

The experiments have shown that phospholipids can be removed from oil by biosurfactant solutions with efficiency up to about 99%, what corresponds to 2-3 mg of residual phosphorus in 1 kg of oil (Klosowska-Chomiczewska, 2011). The pH value of solution do not affect the efficiency of phospholipids removal at biosurfactant concentration 2.5 g/l (Fig.1B) and only a little at concentration 5.0 g/l at pH 13,6, where phosphorus removal is a little lower (88% at X=0.05) than at other pH values (e.g. at pH 5.7 – 99%) (Fig.1A). Moreover, in the case of lower biosurfactants concentration (2.5g/l) the efficiency of degumming is high, even for the lowest X ratio (0.002), while at higher concentration (5g/l) it reaches only 30-50%.

Typical crude rapeseed oil contains 300-500 mg of P in kg of oil (Przybylski et al., 2005). In table 3 there is a comparison of different degumming methods.

Table 3. Comparison of degumming methods efficiency

Degumming method	Residual phosphorus content [mg/kg]	Characteristics of the method (comments)	Ref.
Water degumming	100-200	Removes HP only	Przybylski et al., 2005
Water degumming with acid pretreatment	5-50	Removes HP and NHP but they are carbonized	
Degumming with biosurfactants solution	2-3	We assume that both, HP and NHP are removed in non-invasive way, thus we have a chance to recover them from sludge	Klosowska-Chomiczewska, 2011

CONCLUSIONS

Biosurfactants are less toxic and more easily biodegradable than synthetic surfactants. Moreover they are very effective in different applications including oil refining process, thus they are considered as very promising and prospective biotechnological product. Application of biosurfactants instead of synthetic surfactants in many branches allows to fulfil more and more restrictive environmental expectations and simultaneously ensures very good efficiency.

Biosurfactants effectiveness in crude oil degumming was very high and it reaches up to 99%. The pH of washing solution did not affect efficiency of degumming significantly. Only at pH 13.6 the biosurfactant solution in concentration 5.0g/l reveal lower phosphorus removal than in other pH conditions. The amount of solution added to oil (X ratio) influenced the phosphorus removal rate only in the case of solution of concentration 5.0 g/l, where e.g. for X=0.002 the efficiency was about 30-50% only, instead of 99%.

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