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Full Length Research Paper

Production of a rhamnolipid-type biosurfactant by *Pseudomonas aeruginosa* LBM10 grown on glycerol

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The work herewith investigated the effect of the culture medium composition on rhamnolipid production by *Pseudomonas aeruginosa* LBM10, previously isolated from an estuarine environment in Southern Brazil. Experimental design and surface response methodology were used in order to improve biosurfactant production using glycerol, a renewable carbon source. The assays were carried out in a rotary shaker at 30°C and 180 rpm for 120 h and the parameters studied were glycerol concentration, C/N (carbon/nitrogen) and C/P (carbon/phosphorus) ratios. Low glycerol concentration and a phosphorus-limiting condition were favorable for rhamnolipid production. Contour plots constructed by predictive polynomial equations led to a glycerol concentration of 13.2 g/l, a C/N ratio of 12.8 and a C/P ratio of 40 in order to maximize rhamnolipid concentration (4.15 g/l) associated with a high emulsification index (61%).

Key words: Biosurfactant, surface-active compounds, experimental design, phosphorus limitation.

INTRODUCTION

Petroleum is the main energy source used in the world, but its availability is limited and the search for new renewable energy sources is a major priority. Biofuels such as ethanol and biodiesel are among the most promising sources for the substitution of fossil fuels. Due to the fact that biodiesel produced from animal fats and vegetable oil production generates about 10% (w/w) glycerol as the main by-product and also to its mandatory application on a large commercial scale in many countries, the excess glycerol generated may become an environmental problem, since it cannot be disposed of in the environment (Silva et al., 2009).

Therefore, the enhancement of glycerol through technological means becomes an important aspect for the sustainable production of biodiesel. Studies have presented the production of lipids (Papanikolaou et al., 2008), 1,3 propanediol (Papanikolaou et al., 2008), carotenoid (Razani et al., 2007), citric acid (Levinson et al., 2007), succinic acid (Lee et al., 2001) and rhamnolipid-type biosurfactant (Santa Anna et al., 2002; Wu et al., 2008) using glycerol as the main carbon source.

Biosurfactants are amphiphilic compounds (containing both hydrophobic and hydrophilic portions) produced by microorganisms that reduce the free energy of the system by replacing the higher energy bulk molecules at an interface. They contain a hydrophobic portion with low affinity for the bulk medium and a hydrophilic group that is attracted to the bulk medium (Mulligan et al., 2001).

Bacteria of the genus *Pseudomonas* are known to produce a glycolipid surfactant containing rhamnose and 3-hydroxy fatty acids. The rhamnolipids produced by *Pseudomonas aeruginosa* have been widely studied and are reported to be a mixture of the homologous species RL1 (Rha $C_{10}C_{10}$), RL2 (Rha C_{10}), RL3 (Rha_2 $C_{10}C_{10}$) and RL4 (Rha_2 C_{10}) (Costa et al., 2006).

Although rhamnolipid is an effective biosurfactant and is well suited for applications in bioremediation of oil pollutants, the major hurdle for commercial application of the biosurfactant has been the low yield and high

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production cost (Wu et al., 2008).

Literature indicates that carbon sources, nitrogen sources and C/N ratio (Santa Anna et al., 2002; Rashedi et al., 2006; Wu et al., 2008; Abouseoud et al., 2008) usually play a critical role in the performance of rhamnolipid production by *P. aeruginosa* strains. On the other hand, studies involving the influence of C/P ratio are rare. Factorial design and response surface methodology are important tools to optimize the biotechnological process. Statistically designed experiments are very effective because the affecting parameters can be evaluated collectively, even with a limited number of experiments (Manera et al., 2008). However, the combined effect of several nutrients on biosurfactant production has received little attention (Amézcua-Vega et al., 2007; Wu et al., 2008).

In a previous study, *P. aeruginosa* LBM10, isolated from a southern coastal zone in Brazil, presented good potential for the production of a rhamnolipid-type biosurfactant. The microorganism showed the ability to produce a biosurfactant from different cheap carbon sources, such as soybean oil, soybean oil soapstock, fish oil and glycerol, available in the South of Brazil. A nitrogen-limiting condition (C/N ratio of 100) was favorable for biosurfactant production using soybean oil and sodium nitrate as sources of carbon and nitrogen, respectively, reaching 1.42 g/l. The formation of stable emulsions was better in saline concentrations below 0.5%, pH values in the range of 6 to 9 and temperatures in the range of 35 to 40°C (Prieto et al., 2008).

The effect of culture medium composition on rhamnolipid concentration and emulsifying activity were examined in this study in order to establish the best conditions (glycerol concentration, C/N and C/P ratios) for the rhamnolipid production by *P. aeruginosa* LEBM10 using glycerol as the sole carbon source.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *P. aeruginosa* LBM10, which was previously isolated from a southern coastal zone in Brazil and was able to produce rhamnolipid-type biosurfactant from glycerol (Prieto et al., 2008).

Shaken flasks cultivation

The strain was activated in tryptic soy agar (TSA) tubes and then cultivated at 30°C for 48 h. Bacterial growth was scraped from each slant tube with the aid of 2 ml of 0.1% peptone diluent, and the suspensions from four tubes were inoculated into 500 ml flasks with cotton plugs that contained 200 ml of the medium, incubated in a rotary shaker (Tecnal TE-420, Brazil) at 30°C and 180 rpm for an overall culture period of 120 h. Samples were taken at regular intervals and centrifuged at 3,000 rpm for 30 min. The supernatants were used to determine the emulsification index (E_{24}), rhamnolipid concentration, glycerol concentration and pH, and the pellets were used to quantify the biomass. The medium was made up of glycerol, $NaNO_3$, KH_2PO_4 (variable concentrations according to the experimental design) and $MgSO_4.7H_2O$ (0.2 g/l). The initial pH of the broth was adjusted to 6.5 before sterilization.

The method described by Bicca et al. (1999) was used to determine the E_{24} of the culture samples; addition of 2 ml of diesel oil to the same amount of culture supernatant, mixing it with a vortex for 2 min and leaving it to stand for 24 h. The E_{24} is given as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm). Control assays were performed using an unfermented medium instead of the supernatant.

Rhamnolipids expressed as rhamnose (g/l) were measured in the cell-free culture medium as proposed by Santa Anna et al. (2002), using the phenol-sulfuric method (Dubois et al., 1956). The absorbance was measured at 420 nm in a spectrophotometer (Biospectro SP-22, China) with rhamnose as the standard. Glycerol was assessed by the enzymatic-colorimetric method for triglyceride content evaluation, adapted from Santa Anna et al. (2002). Supernatant samples (0.03 ml) were added to 3 ml of reagent (kit Liquiform®, Labtest, Brazil) and were maintained at 37°C for 10 min. The absorbance was measured at 500 nm, with glycerol as standard. The pH of the supernatant was measured by a pH meter (Marte MB-10, Brazil). The biomass was monitored by measuring the absorbance at 600 nm according to Wu and Ju (1998). A calibration curve between OD_{600} and the cell dry-weight concentration (g/l) was first established.

Experimental design

A central composite rotational design (CCRD) and a total of 17 trials (2^3 plus axial points and three replicates at the central point) was used in order to verify the influence of the variables: glycerol concentration, C/N and C/P ratios, presenting the rhamnolipid concentration and E_{24} as responses. Data were analyzed using Statistica 5.0 software (StatSoft, Inc., USA).

RESULTS AND DISCUSSION

Rhamnolipid concentration

Rhamnolipid concentrations obtained for the assays proposed in the experimental design are presented in Table 1. Biosurfactant concentrations varied from 0.05 to 3.77 g/l in 120 h cultivation. The best value for rhamnolipid concentration occurred in trial 11, corresponding to a concentration of glycerol at level -1 (30 g/l), C/N ratio at level -1.68 (12.8) and C/P ratio at level 0 (80). This result represents a 9.7 time increase in rhamnolipid concentration in relation to previous work (Prieto et al., 2008), which used glycerol as a carbon source. Santa et al. (2002) obtained 3.16 g/l rhamnolipid (expressed as rhamnose), using *P. aeruginosa* PA1, glycerol as the sole carbon source (3% v/v) and a C/N ratio of 60.

On the other hand, the yield factors for glycerol to rhamnolipid conversion ($Y_{P/S}$) and biomass to rhamnolipid conversion ($Y_{P/X}$), in trial 11 conditions, were 0.17 and 0.94 g/g, respectively. Santa Anna et al. (2002), using glycerol as the carbon source, nitrate as the nitrogen source and a C/N ratio of 60, reached a $Y_{P/X}$ of 0.80 g/g. The same authors reported 0.13 g/g for $Y_{P/S}$ and 0.70 g/g for $Y_{P/X}$ when ammonium sulphate was used.

Table 1. Coded values, real values (in parentheses) and experimental data obtained from the assays in the central composite rotational design (CCRD).

Trial	Variable			Rhamnolipid (g/l)	E_{24} (%)
	Glycerol (g/l)	C/N	C/P		
1	-1 (20)	-1 (40)	-1 (40)	1.89	65
2	+1 (40)	-1 (40)	-1 (40)	1.36	53
3	-1 (20)	+1 (120)	-1 (40)	1.20	60
4	+1 (40)	+1 (120)	-1 (40)	0.97	52
5	-1 (20)	-1 (40)	+1 (120)	1.24	62
6	+1 (40)	-1 (40)	+1 (120)	1.11	47
7	-1 (20)	+1 (120)	+1 (120)	0.94	52
8	+1 (40)	+1 (120)	+1 (120)	0.05	3
9	-1.68 (13.2)	0 (80)	0 (80)	1.43	59
10	+1.68 (46.8)	0 (80)	0 (80)	1.19	63
11	0 (30)	-1.68 (12.8)	0 (80)	3.77	61
12	0 (30)	+1.68 (147.2)	0 (80)	1.05	0
13	0 (30)	0 (80)	-1.68 (12.8)	0.58	67
14	0 (30)	0 (80)	+1.68 (147.2)	0.33	0
15	0 (30)	0 (80)	0 (80)	0.43	61
16	0 (30)	0 (80)	0 (80)	0.57	68
17	0 (30)	0 (80)	0 (80)	0.44	58

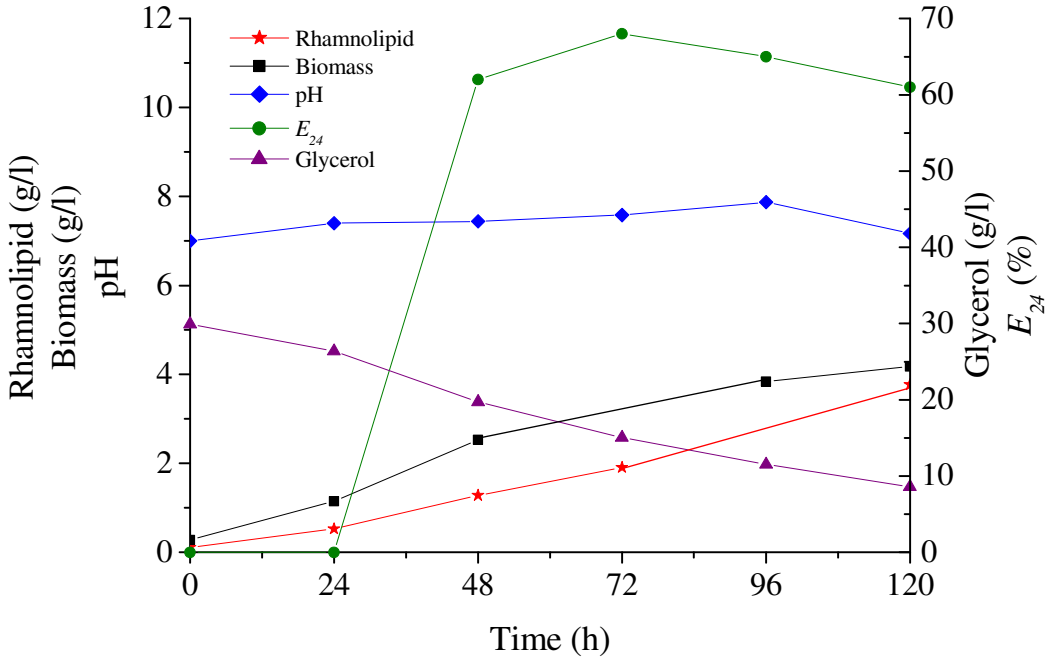

Figure 1. Kinetic profile of *P. aeruginosa* LBM10 growing on glycerol at 30°C and 180 rpm (glycerol concentration = 30 g/l, C/N = 12.8, C/P = 80).

The kinetic profile of this assay is presented in Figure 1. A direct relationship between rhamnolipid production, cell growth and glycerol utilization was observed during the production of biosurfactant by *P. aeruginosa* LBM10 in this condition. However, it cannot be stated that rhamnolipid production by *P. aeruginosa* LBM10 is growth associated, because in the majority of the assays, significant amounts of biosurfactants were produced during the stationary phase (data not shown). In these cases, the behavior can be related to nitrogen depletion

Table 2. ANOVA for rhamnolipid concentration (Rha) and E_{24}.

Source of variation	Sum of squares		Degrees of freedom		Mean squares		F-test	
	Rha	E_{24}	Rha	E_{24}	Rha	E_{24}	Rha	E_{24}
Regression	9.44	5981.60	5	4	1.89	1495.40	12.14	6.25
Residual	1.71	2870.16	11	12	0.16	239.18		
Pure error	0.01	52.67	2	2				
Lack of fit	1.70	2817.50	9	10				
Total	11.15	8851.77	16	16				

$F^{Rha}_{0.95;5;11} = 3.20$; $r^{Rha} = 0.92$; $F^{E_{24}}_{0.95;4;12} = 3.26$; $r^{E_{24}} = 0.82$.

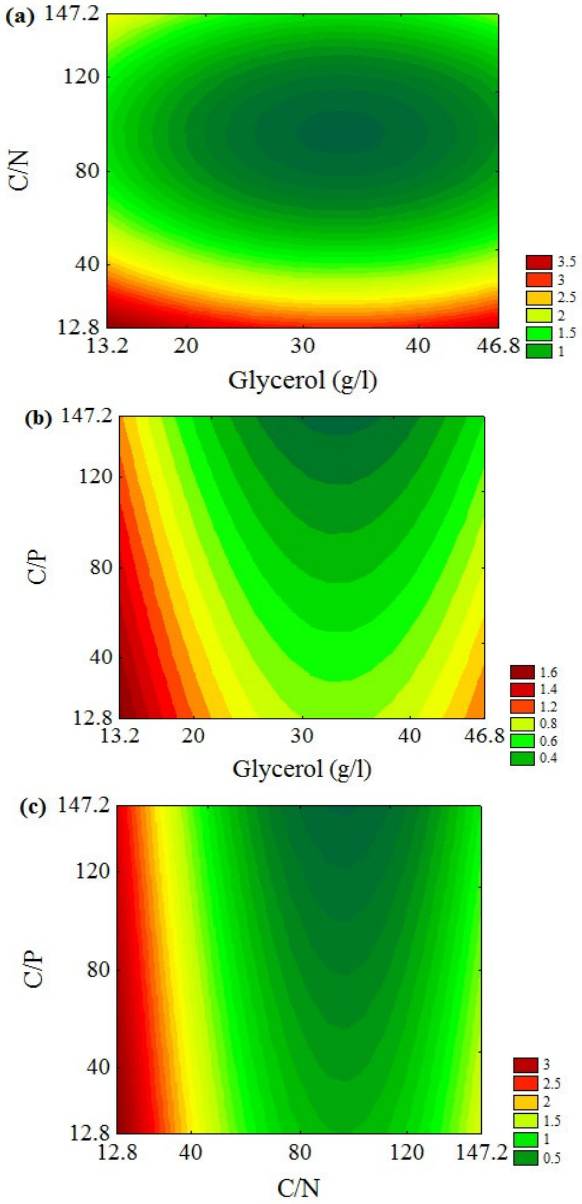

Figure 2. Contour plots for rhamnolipid concentration as a function of (a) glycerol concentration and C/N ratio (C/P = 80), (b) glycerol concentration and C/P ratio (C/N = 80) and (c) C/N and C/P ratios (glycerol concentration = 30 g/l).

throughout cultivation, leading to a nitrogen-limiting condition at the stationary phase that is favorable for biosurfactant production (Desai and Banat, 1997).

A second order model could predict the rhamnolipid concentration (dependent variable) as a function of the glycerol concentration, C/N and C/P ratios. Variance analysis (ANOVA) was used to evaluate fit adequacy. Based on ANOVA, as shown in Table 2, a second order model was established for rhamnolipid concentration. Effects which were not statistically significant were ignored. The correlation coefficient was 0.92 and the F-value was 3.79 times higher than the listed value for a 95% confidence interval. Consequently, the model was found to be adequate to describe the response surface of rhamnolipid concentration. The coded empirical model fitted by regression analysis is given by Equation 1:

$$\text{Rhamnolipid (g/l)} = 0.39 - 0.16\,(\text{Glycerol}) + 0.24\,(\text{Glycerol})^2 - 0.51\,(\text{C/N}) + 0.63\,(\text{C/N})^2 - 0.18\,(\text{C/P}) \quad (1)$$

Figure 2a shows the effects of glycerol concentration and C/N ratio on rhamnolipid concentration. When the C/N ratio was low (12.8) in the entire range of glycerol concentration (13.2 to 46.8 g/l), a rhamnolipid concentration of 3 to 3.5 g/l was obtained. In Figure 2b, higher concentration of rhamnolipid (1.6 g/l) was observed in a low glycerol concentration (13.2 g/l) with a C/P ratio that ranged from 12.8 to 40. It was observed in Figure 2c that a low C/N ratio (12.8) and a C/P ratio which varied from 12.8 to 40 led to a rhamnolipid concentration of 3 g/l. The surfaces indicated that high rhamnolipid concentration can be obtained when using culture medium with a glycerol concentration of 13.2 g/l, a C/N ratio of 12.8 and a C/P ratio of 12.8 to 40.

The C/N ratio is a vital factor influencing the performance of rhamnolipid production and some reports mentioned that rhamnolipid production is more efficient under nitrogen-limiting conditions (Benincasa et al., 2006; Kim et al., 2006). However, optimum values can vary according to the strain and the carbon source (Wu et al., 2008), and a suitable supply is required. Guerra-Santos et al. (1984) showed maximum rhamnolipid production at a C/N ratio of 16 to 18 and no surfactant production below a C/N ratio of 9, where the culture was not nitrogen

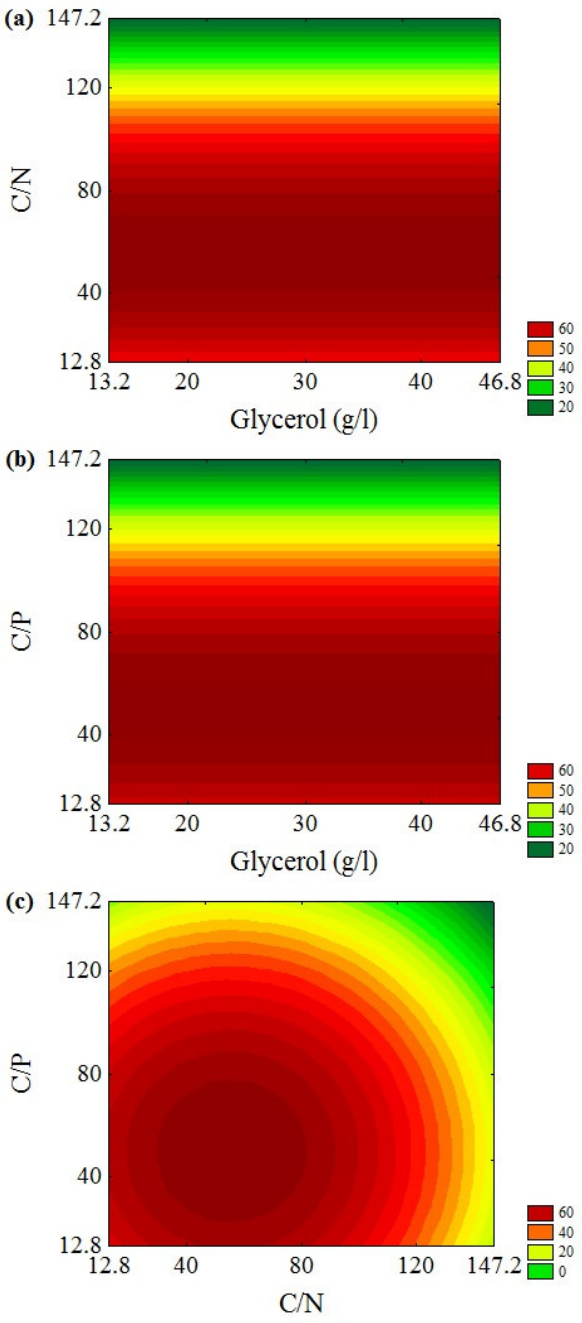

Figure 3. Contour plots for the E_{24} as a function of (a) glycerol concentration and C/N ratio (C/P = 80), (b) glycerol concentration and C/P ratio (C/N = 80) and (c) C/N and C/P ratios (glycerol concentration = 30 g/l).

limited. For *P. aeruginosa* EM1, Wu et al. (2008) observed an optimum C/N ratio of 26 when glucose was used as a carbon source and a C/N ratio of 52 when glycerol was used and a poor rhamnolipid performance was obtained when C/N ratio was higher than 52. A lower C/N ratio (10) was favorable for biosurfactant production by *P. fluorescens* Migula 1895-DSMZ (Abouseoud et al., 2008).

The expression of the rhamnolipid in *P. aeruginosa* is coordinately regulated at the transcriptional level by quorum-sensing systems which are dependent on cell-density and regulators that respond presumably to a variety of signals. According to Bazire et al. (2005), phosphate limitation is a signal that positively affects rhamnolipid production. In agreement, Guerra-Santos et al. (1984) and Rashedi et al. (2006) observed that a C/P ratio of 16 resulted in maximum rhamnolipid production. *P. aeruginosa* LBM10 clearly demonstrated a biosurfactant production in this work, which was favored by a phosphorus-limiting condition.

E_{24}

The results of the E_{24} in the CCRD for the three studied variables are presented in Table 1. It was observed that in some conditions, emulsification activity was not observed (trial 12 and 14) or a poor emulsion was formed (trial 8). The best results (E_{24} close to 70%) were similar to Ilori et al. (2005) for *Aeromonas* spp. growing on crude oil and to Abouseoud et al. (2008) for *P. fluorescens* Migula 1895-DSMZ. These values represent an increase of 1.5 times in relation to previous work using glycerol as the carbon source (Prieto et al., 2008).

Based on the analysis of variance (ANOVA), as show in Table 2, a second order model was established for the E_{24}. Effects, which were not statistically significant, were not considered. The correlation coefficient was 0.82 and the F-value was 1.9 times higher than the listed value for the 95% confidence interval. A second order polynomial equation was generated (Equation 2) in order to describe the E_{24} as a function of the studied variables:

$$E_{24}(\%) = 63.67 - 11.91\ (C/N) - 9.74\ (C/N)^2 - 13.09\ (C/P) - 8.68\ (C/P)^2 \quad (2)$$

Figure 3 presents the contour plots for E_{24}. Figure 3a shows that the best emulsifying properties (E_{24} around 60%) were obtained at a C/N ratio of 40 to 70, for all glycerol concentrations (13.2 to 46.8 g/l). In Figure 3b, it is possible to see that the use of a C/P ratio from 40 to 80 for the entire range of glycerol concentration, the same E_{24} index was obtained. In Figure 3c, the maximum E24 obtained was 70%, when a C/N ratio from 35 to 80 and a C/P ratio from 20 to 80 were used.

Conclusion

This work demonstrated the biosurfactant-producing potential of an indigenous *P. aeruginosa* LEBM10 isolate capable of producing rhamnolipid effectively from glycerol as a carbon source. The variables glycerol concentration, C/N ratio and C/P ratio were found to play a crucial role in rhamnolipid concentration and E_{24}. Experimental design and response surface methodology were shown to be an

effective tool for the improvement of medium composition leading to a higher rhamnolipid concentration and E_{24}. Contour plots constructed by predictive polynomial equations led to a glycerol concentration of 13.2 g/l, a C/N ratio of 12.8 and a C/P ratio of 40 in order to maximize rhamnolipid concentration (4.15 g/l) while obtaining a high E_{24} (61%). This rhamnolipid concentration represents a 10 times increase in relation to previous work.

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