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Acid Hydrolysis of Pectin for Cell Growth of *Cupriavidus necator*

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Abstract: The effect of acid concentration (1-7%, v/v) and temperature (70-100°C) on the pectin hydrolysis was studied using pectin at 1% (w/v) for 4 h hydrolysis. Experiments were arranged according to a central composite statistical design and Response Surface Methodology (RSM) was used to assess factor interactions and empirical models on response variable (reducing groups concentration - RGC). The highest RGC (6.5 g L^{-1}) was achieved at 100°C and 1% (v/v) H_2SO_4. Kinetics of RGC release was also performed and hydrolysate was used as carbon source for cell growth of *Cupriavidus necator*. The maximum specific growth rate and the substrate-to-cell conversion factor were 0.26 h^{-1} and 0.55 g g^{-1}, respectively an indicative of that pectin hydrolysate is a potential substrate for cell growth of this microorganism.

Key words: Pectin, *Cupriavidus necator*, acid hydrolysis, experimental design, reducing group concentration

INTRODUCTION

Cupriavidus necator (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*) was originally developed as a source of Single Cell Protein (SCP) in the 1970's (Reinecke and Steinbuchel, 2009) for animal feeding and also for humans. Actually, it is the microorganism that has been most extensively studied for fermentative production of polyhydroxyalkanoates (PHAs).

These biopolymers are polyesters of carboxylic acids, synthesized as intracellular compounds and energy storage materials for more than a hundred species of microorganisms (Lai *et al*., 2005). Accumulation of PHA by *C. necator* can be as high as 80% of its cell dry weight, using different sources of substrate such as glucose, fructose, vegetable oils and oils waste frying (Obruca *et al*., 2010), among other sources.

PHAs commercial applications have been limited by their high price, making them more expensive than synthetic plastics (Poirier *et al*., 1995). High costs of raw material used as a carbon source (Kim, 2000) can represent about 40% of total cost of production and use of agro-industrial residues as cheap carbon sources is an alternative in order to reduce the production cost of biopolymers.

Development of intensive production methods for citrus crops, its high availability and high quantity of material processed from juice production has attracted the attention to the problems of this agroindustrial waste management. Orange peel present a great potential to be used as substrate for the growth of many microorganisms for obtaining products of high added value (Rivas *et al*., 2008).

Pectins are structural polysaccharides found in different amounts and compositions (May, 2000) and consisting mainly of galacturonic acid and neutral sugars such as rhamnose, galactose, arabinose and xylose (Yapo *et al*., 2007). Several treatments have been proposed for the hydrolysis of pectic substances, including acids and enzymatic methods (Garna *et al*., 2006). Pectin hydrolysis products have several biotechnological applications in agriculture, medicine (Bedouet *et al*., 2005), food industry and can be used as substrates for fermentation purposes. Additionally, further research is required to understand mechanisms of pectin hydrolysis.

There are no reports in the literature using pectin or pectin hydrolysate as carbon source for cell growth of *C. necator* aiming PHAs production. The purpose of present investigation was to evaluate the acid hydrolysis of commercial pectin, using experimental design and Response Surface Methodology (RSM) in study of variables that play a very significant role in enhancing the acid hydrolysis of pectin, such as acid concentration and temperature. Pectin hydrolysate was used as substrate for cell growth in a submerged culture of *C. necator*.

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MATERIALS AND METHODS

Pectin: Commercial citric pectin was supplied by Vetec®. Pectin esterification degree was determined according to Bochek *et al.* (2001). Tests were carried out in triplicate.

Acid hydrolysis: Hydrolysis reactions were performed using 5 g pectin and 500 mL H_2SO_4 (1% w/v) in system reflux using a rotaevaporator (Marconi®), at a reaction time of 4 h. Nine experiments were conducted using a central composite statistical design (2^2) for the study of linear and interaction effects of the two factors (acid concentration and temperature) on dependent variable (reducing groups concentration-RGC). Experiments were performed randomly and centre values for factors were repeated in five experiments for error estimation. Levels of the factors studied and experimental conditions are shown in Table 1.

Software Statistica 7.0 (Statsoft®) was used to fit the first-order model to the independent variables at a confidence level of 95% and to determine the region to maximize the reducing groups concentration. Selected condition was repeated in triplicate and the hydrolysis kinetics was monitored by taking samples at defined time. Samples were immediately neutralized using NaOH 50% (w/v), cooled in ice bath and diluted for RGC and sugars and galacturonic acid analysis by HPLC. Further, hydrolysate suspension was used as carbon source for cell growth of *C. necator*.

Microorganism and culture maintenance: *C. necator* was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and maintained in the culture collection of the Antibiotics Department of the Federal University of Pernambuco, Brazil (*C. necator* UFPEDA 0604).

Culture media: Nutrient Broth (NB) medium was used for inoculum development. In second step, Mineral Medium (MM), based on work by Ramsay *et al.* (1990), modified by Aragao *et al.* (1996), was used for cellular growth. Carbon sources tested were galacturonic acid at 1.5% (w/v) and acid hydrolysate.

Table 1: Levels of the factors and experimental conditions used in the experimental design (central composite statistical design 2^2)

	Experimental factors			
Run	Temperature (°C)		H_2SO_4 (% v/v)	
1	+1	100	+1	7
2	-1	70	+1	7
3	+1	100	-1	1
4	-1	70	-1	1
5-9	0	85	0	4

Culture conditions: Inoculum was prepared from transferring one slant into NB medium and the microorganism was grown at 300 rpm, 30°C for 24 h. Samples were taken to determine pH and cell optical density at 600 nm. When microorganism reached the exponential growth phase (10 h cultivation), culture was transferred into 250 mL Erlenmeyer flasks using 47.5 mL MM medium containing acid hydrolysate or galacturonic acid at 1.5% (w/v). Inoculum size was 5% (v/v) and flasks were incubated at same conditions. Samples were taken to determine cell optical density, RGC and pH.

Analytical methods: Cell dry weight was assessed by spectrophotometry at 600 nm, based on standard calibration curve for cell optical density as a function of cell dry weight. pH was monitored by potentiometry and RGC was accomplished in the cell-free filtrate by the 3, 5 dinitrossalicilic acid method according to Miller (1959), using glucose as reference for the preparation of the calibration curve.

Sugars and galacturonic acid concentrations were determined by HPLC (Varian) equipped with refractive index detector. It was used a column for organic acids (Aminex HPX-87H, 300×780 mm, Bio Rad), at 65°C, mobile phase an aqueous solution of H_2SO_4 8.0 mM and flow rate of 0.6 mL min^{-1}. Standard solutions of galacturonic acid, glucose, fructose, galactose, xilose and arabinose (Sigma®) were prepared in the range of 0.2 to 10 g L^{-1} for comparison of peaks retention times.

RESULTS

Degree of pectin esterification (DE) was 67.3±1.0%, indicating a high degree of esterification.

The response surface for RGC was drawn as a bi-dimensional plot (Fig. 1a) and shows that by increasing the temperature and decreasing the acid concentration the RGC was increased, achieving the maximum value of 5.9 g L^{-1} for a H_2SO_4 concentration of 1% (v/v) at 100°C. Linear mathematical model was built (Eq. 1) and R^2 value was corresponding to 0.99. Interactive temperature-H_2SO_2 term was discarded because presented p-value higher than 0.05 ($p = 0.054$) at 95% confidence level. p-values for temperature and H_2SO_2 variables were 0.00436 and 0.0005, respectively.

$$RGC = 0.9273 - (0.0894 \times H2SO4) + 0.0560 \times T \quad (1)$$

As shown in Pareto graph (Fig. 1b), on the contrary of temperature, an increase in acid concentration has a negative effect on the variable response. The experimental findings were in close agreement with the prediction

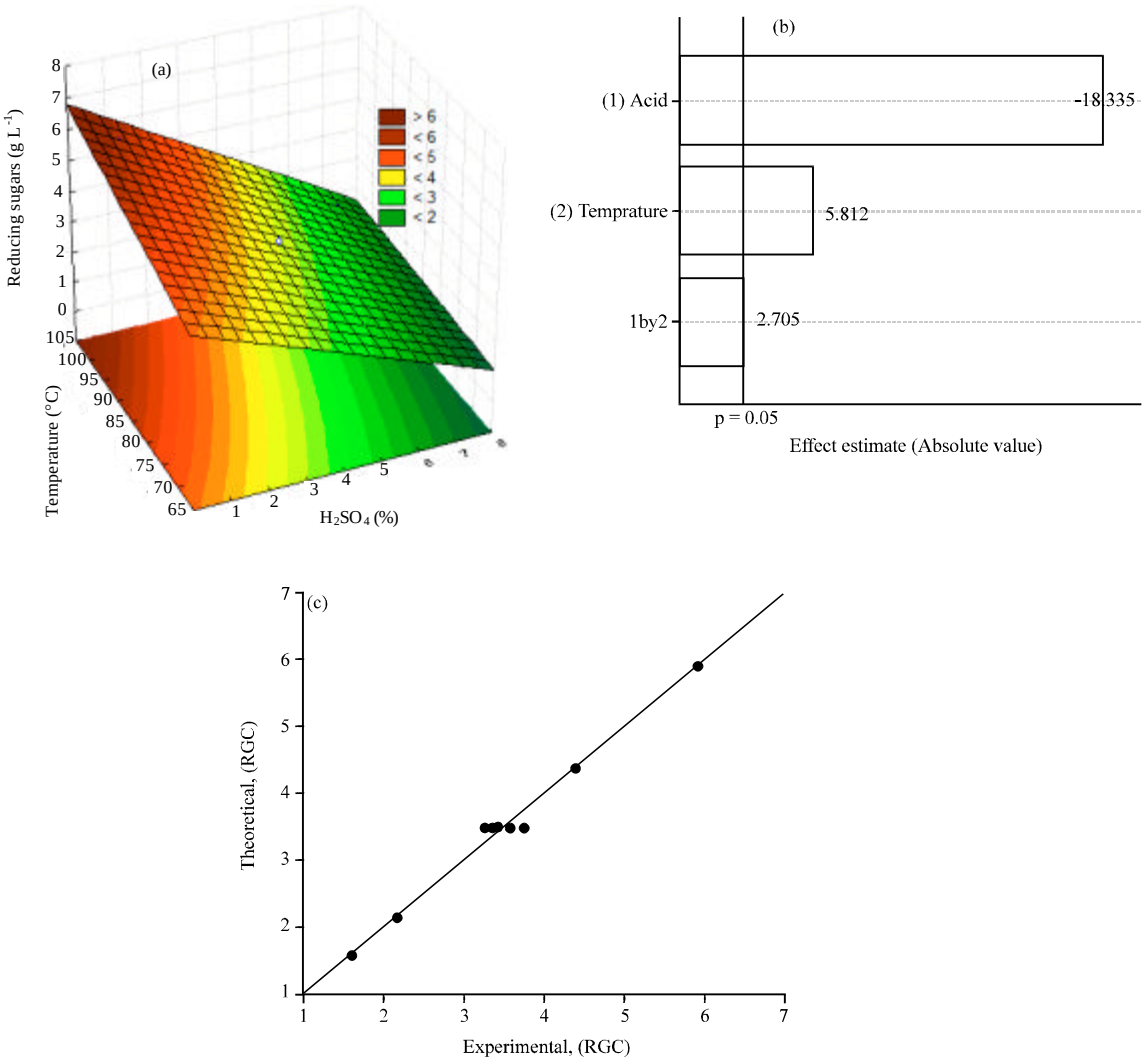

Fig. 1(a-c): (a) Response surface, (b) Pareto diagram and (c) correlation between theoretical and experimental reducing groups concentration values (RGC) as a function of temperature and H_2SO_4

model in view of the satisfactory correlation between theoretical response dates and experimental values (Fig. 1c).

Results indicate there is an optimum region for hydrolysis ratio at high level of temperature and low level of acid concentration. Considering equipment limitations to promote temperatures above 100°C, experimental conditions for determining kinetics of the reducing groups released were established at 1% (v/v) and 100°C for H_2SO_4 concentration and temperature, respectively.

Monitoring hydrolysis kinetics is shown in Fig. 2, with high reducing compounds liberation in the first 15 min, followed by a gradual increase up to 4 h. At the end of hydrolysis the average RGC is around 6 g L^{-1}, corresponding to a yield hydrolysis of 60% (Fig. 2a).

Two groups were evaluated in the hydrolysate chromatogram-galacturonic acid+glucose and fructose +galactose+xylose. For comparison from the standard chromatogram, each group presented a single peak and thus the total area of each peak represents the sum of the compounds concentrations. Arabinose concentrations were below 0.2 g L^{-1} in all samples analyzed.

The release profiles of hydrolysate components were consistent with the results of RGC, with a high initial release rate for both groups. After 2 h was observed a decline of fructose, galactose and xylose content and from 4 h this decline was accentuated for both groups (Fig. 2b).

Figure 3 shows cell growth and pH variation during *C. necator* cultivation in nutrient broth medium with respect to inoculum development. Microorganism

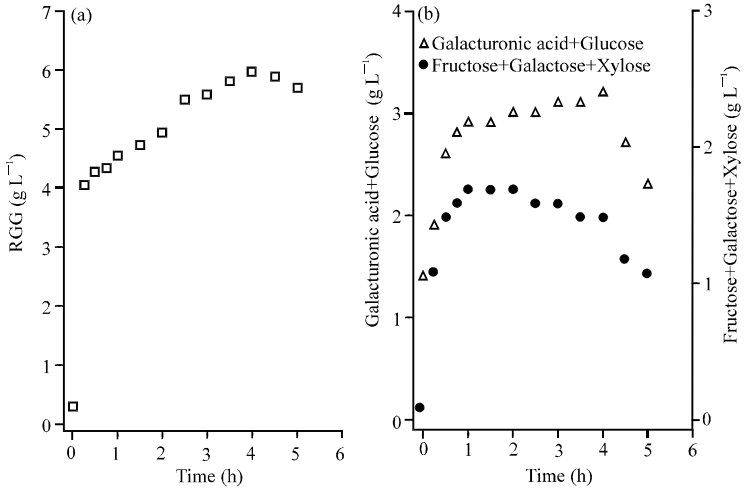

Fig. 2(a-b): Reducing Groups Concentration-RGC (a), Galacturonic acid and Glucose (b) and Fructose, Galactose and Xylose (c) concentrations during pectin hydrolysis at 100°C and 1% (v/v) H_2SO_4 (pectin concentration 1% w/v)

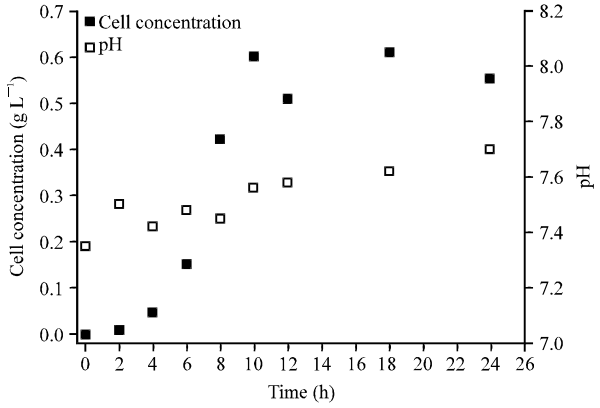

Fig. 3: Cell growth of *C. necator* and pH variation during the 24 h of cultivation in nutrient broth (NB)

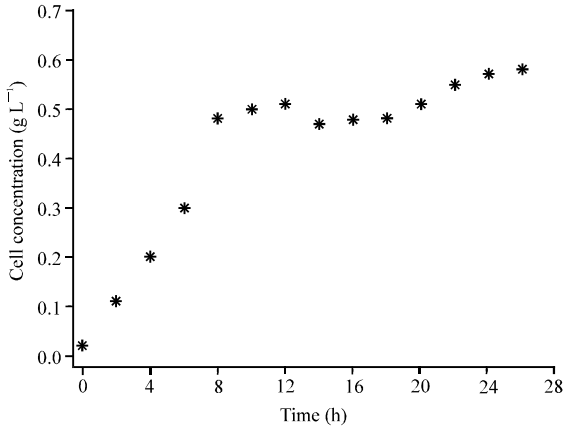

Fig. 4: Cell growth of *C. necator* during cultivation in MM medium containing 1.5 % (w/v) galacturonic acid

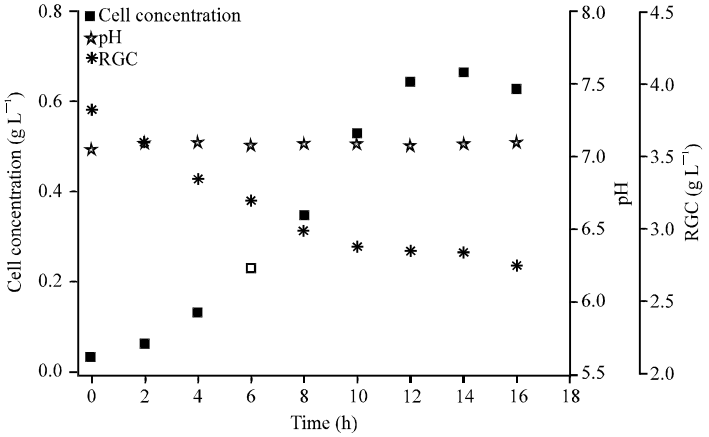

Fig. 5: Cell concentration, pH variation and Reducing Groups Concentration-RGC during *C. necator* cultivation in MM medium supplemented with acid hydrolysate

presented a lag phase of 4 h and exponential growth until 10 h, with maximum growth rate (μ_{max}) of 0.49 h^{-1}. pH values increase with the cellular growth. Thus, period of 10 h was used as criteria for the transfer of inoculum.

In order to verify if galacturonic acid is used as carbon source for cell growth, experiments were conducted using MM medium containing this compound at a concentration of 1.5% (w/v) (Fig. 4). *C. necator* grows without lag phase and at an arithmetic rate until 8 h. Using mineral medium containing acid hydrolysate, cell growth occurs immediately after inoculation of the cells with exponential growth up to 10 h and μ_{max} of 0.26 h^{-1} (Fig. 5).

pH values were around 7.0 and initial concentration of reducing groups was 3.82 g L^{-1}. High substrate consumption rate was observed until 10 h, concomitantly with the exponential growth phase. Final cell concentration was corresponding to 0.63 g L^{-1} and the substrate-to-cell conversion factor ($Y_{X/S}$) was 0.55 g g^{-1}. After 16 h cultivation a residual reducing groups concentration of 2.8 g L^{-1} was obtained.

DISCUSSION

Pectin showed high degree of esterification, for according to May (2000), pectins are divided into two classes: pectins with high degree of esterification (>50%) and low degree of esterification (<50%). Esterification degree represents the percentage of acid group present in the form of ester and is an important parameter to be considered in hydrolysis study. Krall and Mcfeeters (1998) investigated pectins hydrolysis at pH 3.0 and 100°C, demonstrating that an increasing degree of pectin esterification leads to a reduction in the hydrolysis rate and a slower release of reducing groups.

An increase in acid concentration has a negative effect on the variable response. Smidsrod *et al.* (1966) reported that in strongly acidic conditions (pH<1.0) polipectate was more slowly hydrolysed to neutral polysaccharides compared with milder conditions of acidity (pH 2.5-4.5). Garna *et al.* (2006), studying kinetics of pectin hydrolysis at different sulfuric acid concentrations, obtained an optimal concentration around 1 M. Concentrations of 0.2 and 2 M had lower hydrolysis rates. The same authors investigated higher percentage of galacturonic acid was obtained at 100°C. Leitao *et al.* (1995) reported similar results when studied different acids and temperatures on the hydrolysis of sunflower pectin.

According to Grohmann *et al.* (1994), an advantage dilute acid hydrolysis has over enzymatic treatment is the higher rate of depolymerization and solubilization of polysaccharides. Treatment with acids can be used to increase the efficiency of subsequent enzymatic hydrolysis and improve the Liquefaction rate of concentrated peel slurries. On the other hand, it is a less sensitive process than enzymatic hydrolysis, because the hydronium ion is a less selective catalytic agent for this reaction.

Kinetics of hydrolysis showed high initial release of reducing groups can be explained considering that commercial pectins with a high esterification degree are generally extracted in hot acidic conditions, thus, regions containing high proportions of neutral sugars are hydrolyzed (May, 2000), forming a soluble fraction of the pectin. In addition, the initial conditions of acidity and temperature are sufficient to break glycosidic bonds more susceptible to hydrolysis. According to Novosel-skaya *et al.* (2000), resistance to acid hydrolysis of glycosidic linkages in pectin occurs in the following order: GalA α(1-4) GalA>GalA α(1-2) Rha>Rha α(1-4)

GalA>neutral sugar α(1-4) neutral sugar. This resistance variation explains the gradual release of reducing groups by the end of the hydrolysis process.

Garna *et al*. (2006) investigated hydrolysis of high esterification degree pectin using different concentrations of sulfuric acid at 100°C and quantifying the galacturonic acid monomers released. Authors observed a gradual release in the beginning of hydrolysis in all treatments but a decline in the free galacturonic acid concentration was observed after 2, 6 and 18 h for sulfuric acid concentrations of 2, 1 and 0.2 M, respectively suggesting that hydrolysis can result in destruction rates of free galacturonic acid higher than polymer liberation rates. One possible reason is that the galacturonic acid is subject to degradation forming lactones (Blake and Richards, 1968) or furfural (Medina *et al*., 1942).

Garna *et al*. (2004) showed a similar effect on the release of pectin neutral sugars and its susceptibility of degradation. These authors observed the beginning of degradation from 2 h to xylose and glucose and 3 h to galactose, using sulfuric acid 1 M at 100°C. For sulfuric acid concentration at 2 M, at the same temperature, the onset of degradation was observed from 1 h for all sugars except rhamnose.

Pentose components of polysaccharide are generally more easily liberated than the hexoses but the released pentose sugar is more liable to destruction during acid hydrolysis. Furanic compounds can be produced from sugars degradation products: furfural, from the degradation of pentoses (xylose and arabinose) and 5-hydroxymethylfurfural (HMF), originated from the degradation of hexoses (glucose, mannose and galactose).

Furfural and HMF are usually considered toxic to many organisms, such as mammalian cells (Janzowski *et al*., 2002), fungi (Szengyel and Zacchi, 2000), yeast (Taherzadeh *et al*., 1999) and bacteria (Zaldivar *et al*., 1999). The toxic effect of these compounds seems to be associated to its high reactivity to lipids, proteins and nucleic acids, besides being able to cause damage to the cell wall. Results obtained in the present work suggest that until 4 h of hydrolysis the formation of degradation products was low. Thus, hydrolysate obtained could be suitable for cell growth of *C. necator*.

There are no reports in the literature on the use of galacturonic acid and pectin hydrolyzed for cell growth of *C. necator*. Wei *et al*. (2011) studied a system for screening production strains in order to optimize PHA production in *C. taiwanensis*. Different carbon sources were evaluated on PHB synthesis and galacturonic acid was used for cell growth but had a negative effect on biopolymer production.

Dalcanton *et al*. (2010) studied the same cultivation conditions used in this work using hydrolyzed rice starch and obtained similar results for μ_{max} (0.22 and 0.24 h^{-1}). Similar values were also observed by Marangoni *et al*. (2001) (0.26 h^{-1}) when investigated use of invert sugar as carbon source and Kim *et al*. (1994) (0.20 h^{-1}), when used glucose. Thus, results of this work indicate that pectin hydrolysate is a potential substitute for glucose for cell growth of *C. necator*.

The substrate-to-cell conversion factor ($Y_{X/S}$) obtained in this work (0.55 g g^{-1}) is comparable to the theoretical value suggested for *C. necator* (0.5 g g^{-1}), as related by Repaske and Repaske (1976). After 16 h cultivation a residual reducing groups concentration of 2.8 g L^{-1} was obtained which was also observed in other studies for various carbon sources, such as molasses, acetate and glucose (Baei *et al*., 2009).

CONCLUSION

The application of response surface methodology can be a practical useful tool for optimization of reaction parameters for the enhanced yields of RGC during acid hydrolysis of pectin. By monitoring the kinetics of hydrolysis, it was shown that there is a gradual increase of RGC released, indicating to be a suitable method for conversion of peel carbohydrates to monomeric sugars with low degradation in inhibiting compounds. We have shown that *C. necator* has ability to consume the products of pectin hydrolysis which demonstrates that utilization of waste pectin is a promising and economic strategy aiming PHAs production.

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