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Comparison of acid and enzymatic hydrolysis of pectin, as inexpensive source to cell growth of *Cupriavidus necator*

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Abstract: The present work investigated what the appropriate methods of hydrolysis of pectin for reducing compounds (RCs) production, employed as a substrate for cell growth of *Cupriavidus necator*. This microorganism has great importance industrial, because besides potential single cell protein (SCP), is the most studied microorganism for production of polyhydroxybutyrate (PHB), and both processes require high cell concentration with inexpensive substrates For this, it was compared to acid and enzymatic hydrolysis procedures, through rotational central composite experimental design, using pectin concentration (1.0%). It was analyzed as a variable response for both experimental design, the RCs' production. The best conditions of each procedure were used in study kinetics of RCs' production and as a substrate for cell growth of *C. necator*. The results indicated that the enzymatic hydrolysis method was the most efficient, with a 93.0% yield of RCs, while the yield for acid hydrolysis was 60.0%. The optimum conditions for enzymatic hydrolysis were an enzyme concentration of 10.01 UI/g (International Unit of enzyme per gram of pectin) and an agitation speed of 230.3 rpm. *C. necator* showed satisfactory growth in the media containing pectin hydrolysates, with specific growth rates (μMax) similar to those reported for other substrates.

Key words: *Cupriavidus necator*, galacturonic acid, pectin depolymerization, pectin hydrolysates, polygalacturonase.

INTRODUCTION

Pectins are among the most abundant natural polysaccharides, present as a component of the primary cell wall and middle lamella of fruits and vegetables and normally found associated with cellulose, hemicellulose, and lignin. Although ubiquitous in almost all plants, pectins of the citrus, apple, sugar beet, and sunflower are considered of special interest, due to the physicochemical quality and the availability of their biomasses in agroindustrial wastes (Muzzarelli et al. 2012, Adetunji et al. 2017).

Pectins are high molecular weight heteropolymers, with a high content of galacturonic acid (GalA) – an oxidized form of *D*-galactose, which constitutes the main monomeric unit (around 65.0%) of the pectin molecule. The structure of pectins can change according to the material

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of origin, and its understanding is currently characterized by much speculation and has not yet been fully resolved. A general model proposes linearly alternating chains of homogalacturonans, composed of repeating units of (1→4)-α-D-GalA, interrupted by branched regions composed of (1→2)-α-L-rhamnose units, to which are bound neutral sugars including galactose, arabinose, xylose, and fructose (May 2000, Caffall and Mohnen 2009).

Pectin can be degraded by either acid or enzymatic hydrolysis. Acid methods are commonly used in analytical procedures; however, divergent results suggest that these techniques still require significant improvement. The maintenance of strongly acidic conditions for prolonged periods can result in a rate of destruction of free GalA that exceeds the rate of polymer release (Garna et al. 2006).

Enzymatic procedures are widely used in industry to improve the yield during the extraction and clarification of juices. The (endo) polygalacturonases (E.C.3.2.1.15) are probably the most important pectinases for biocatalysis, since they are able to hydrolyze both pectin and pectic acids. However, few kinetic studies have been reported concerning the mode of action of these enzymes (Kiss et al. 2008, Kohli and Gupta 2015).

Agroindustrial pectin-rich waste, such as fruit pulp, husks, and bagasse, are potentially suitable feedstocks for bioconversion into products of biotechnological interest. Often bioconversions of these residues can be done by acid or enzymatic hydrolysis to provide a useful source of carbon and energy for use in biotransformation processes (Pinto et al. 2006).

Although few studies have been undertaken concerning the saccharification of pectin, *D*-galacturonic acid is an important primary material in the food, pharmaceutical, and cosmetic industries and can be used to produce vitamin C, acidification agents, and surfactants (Jörneding et al. 2002). As an application alternative, previous work by our research group demonstrated the capability of *C. necator* to grow using GalA as the sole source of carbon as well as using the products of acid hydrolysis of pectin (Locatelli et al. 2011), but optimizing the hydrolysis conditions should be further studied.

C. necator was famous as a potential single cell protein (SCP) in the 1970s, studies evaluated that bacterial cells would average 50.0% protein, with 93.0% of digestibility by animals and high concentration of important amino acids similar to those found in casein. However, the competition from soybased protein resulted in SCP not receiving much attention, but in recent years has resurgence the interest in SCP and PHA as a component of animal feed to increase the metabolizable energy content (Kunasundari et al. 2013).

Although hundreds of species of microorganisms are capable of accumulating polyhydroxyalkanoates (PHAs), *C. necator* is the most important microorganism and has been extensively studied for industrial production of PHAs because it can accumulate up to 80.0% of its dry mass in the form of biopolymers (Akaraonye et al. 2010, Wang et al. 2014). However, the polyhydroxyalkanoates production is still 5 to 10 times more expensive than chemically synthesized polymers, and the substrate may represent more than 40.0% of the cost of production (Akaraonye et al. 2010, Albuquerque and Malafaia 2018).

Thus, either for SCP production or for PHA production is very important to identify a new and inexpensive substrates source that can be used to cell growth of *C. necator*. Like this, the objective this work was to compare methods of acid and enzymatic hydrolysis for the saccharification of pectin and investigate its use as a substrate for the growth of *C. necator*.

MATERIALS AND METHODS

A commercial citrus pectin with a high degree of esterification (67.0%) was purchased (Vetec, Brazil). Sulfuric acid (Vetec, Brazil) was employed in the acid hydrolysis and a polygalacturonase (Sigma-Aldrich, St. Louis, US, E.C.3.2.1.15), with an enzymatic activity of 1.32 UI per mg of enzyme, was used in the enzymatic hydrolysis, the value of enzymatic activity was confirmed experimentally (data not shown).

In order to refine the conditions for pectin hydrolysis and maximize the yield of reducing compounds (RCs), two independent variables (defined separately for each hydrolysis method) were evaluated using a full 2^2 experimental design (rotational central composite design – RCCD), with three central points (level 0) and four axial points (levels ± α, where α = 1.4142), totaling 11 experiments. This experimental design model, it allows a greater comprehension of the parameters tested, minimizing the experiments number. The experiments were performed randomly, and the data were analyzed using Statistica 8.0 software (StatSoft, Dell Software, US), with a 95.0% confidence level. The experimental error was obtained from the mean and standard deviation of the central points. The software calculate an empirical model described by Equation, through the experimental data, allowing prediction of the experimental value at any point within the study area.

ACID HYDROLYSIS

The acid hydrolysis of pectin was based on the method proposed by Wenzel (2001). The tests were performed using a rotary evaporator reflux system (Marconi). The initial pectin concentration was 1.0% (w/v), and temperature and acid concentration were the independent variables (Table I). For this, a 5.0 g portion of pectin was added to 500 mL of a solution of sulfuric acid in a distillation flask, and the mixture was refluxed for 5 hours. At the end of each test, the hydrolysate was cooled in an ice bath and immediately neutralized with NaOH 50.0% (w/v).

The results of the experimental design were used to define the hydrolysis conditions that favored RCs' production. Experiments were then performed in triplicate under these conditions, with samples removed at the start, and then after every 15 minutes during the first hour, and subsequently after every 30 minutes up to a final time of 5 hours. The samples were immediately neutralized with NaOH 50.0% (w/v), cooled in an ice bath, diluted for analysis of RCs, and stored at -18 °C until the chromatographic procedures were performed. The final hydrolysate obtained was neutralized, sterilized in an autoclave, and used as the substrate in the microorganism culture medium.

ENZYMATIC HYDROLYSIS

According to the information provided by Sigma, the polygalacturonase utilized for the enzymatic hydrolysis experiments presented optimum activity at pH 4.0 and a temperature of 50 °C. To enable comparison with the acid hydrolysis method, the same pectin concentration (1.0% w/v) was

TABLE I
Codified levels and actual values of the variables studied in the acid hydrolysis experiments.

Test	Concentration of H_2SO_4 (% v/v)	Temperature (°C)
1	-1 (1.9)	-1 (74.4)
2	+1 (6.1)	-1 (74.4)
3	-1 (1.9)	+1 (95.6)
4	+1 (6.1)	+1 (95.6)
5	-1.41 (1.0)	0 (85.0)
6	+1.41 (7.0)	0 (85.0)
7	0 (4.0)	-1.41 (70.0)
8	0 (4.0)	+1.41 (100.0)
9	0 (4.0)	0 (85.0)
10	0 (4.0)	0 (85.0)
11	0 (4.0)	0 (85.0)

employed, and the independent variables were the enzyme concentration and the agitation speed (Table II).

The tests were performed using 125 mL Erlenmeyer flasks containing 0.5 g of pectin and 50 mL of sodium acetate buffer (50 mM) and incubated under orbital agitation for 24 hours. At the end of the reaction period, the enzyme activity was interrupted by placing the sample in a boiling water bath for 5 minutes.

The optimum conditions for enzymatic hydrolysis were identified from the results, and experiments were then performed in triplicate, with removal of sample aliquots at the start of the experiment, and then after every 15 minutes during the first hour, and subsequently after every hour during a total period of 24 hours. After removal, the samples were immediately placed in a boiling water bath for 5 minutes, diluted for analysis of RCs, and stored at -18 °C until the chromatographic procedures were performed. The final hydrolysate was neutralized, sterilized in an autoclave, and it was used as the substrate in the microorganism culture medium.

TABLE II
Codified levels and actual values of the variables studied in the enzymatic hydrolysis experiments.

Test	Enzyme conc. (UI/g)	Agitation speed (rpm)
1	-1 (6.9)	-1 (211.6)
2	+1 (11.1)	-1 (211.6)
3	-1 (6.9)	+1 (268.4)
4	+1 (11.1)	+1 (268.4)
5	-1.41 (6.0)	0 (240.0)
6	+1.41 (12.0)	0 (240.0)
7	0 (9.0)	-1.41 (200.0)
8	0 (9.0)	+1.41 (280.0)
9	0 (9.0)	0 (240.0)
10	0 (9.0)	0 (240.0)
11	0 (9.0)	0 (240.0)

MICROORGANISM AND MAINTENANCE OF THE CULTURE

The strain of *Cupriavidus necator* was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 545), and maintained in the culture collection of the Department of Antibiotics of the Federal University of Pernambuco (UFPEDA 0604). During the tests, the bacterium culture was frequently subcultured on tubes containing nutrient agar slants and stored in a refrigerator (4–8 °C).

PREPARATION OF THE INOCULUM

For the production of the inoculum, one loopful of the bacterial culture was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing 100 mL nutrient broth (NB) medium. The flask was incubated in a shaking incubator at 30 °C and 300 rpm for 10 hours. This time of cultivation had previously been established for attainment of the exponential growth phase (data not shown).

CULTURE CONDITIONS

The culture was performed under the same conditions described previously, employing an inoculum of 5.0% (v/v) of the cellular material obtained in the previous step. The mineral medium used was described by Ramsay et al. (1990), modified by Aragão et al. (1996). The medium was composed of a mixture of four solutions, as follows (with concentrations as g/L): Nitrolactic acid (0.19), ferrous ammonium citrate (0.06), $MgSO_4.7H_2O$ (0.5), $CaCl_2.2H_2O$ (0.01), solution of oligoelements (1.0 mL) (Solution 1); $Na_2HPO_4.12H_2O$ (8.95), KH_2PO_4 (1.5) (Solution 2); $(NH_4)_2SO_4$ (5.0) (Solution 3); pectin hydrolysate (Solution 4). The solution of oligoelements consisted of (g/L): H_3BO_3 (3.0), $CoCl_2.6H_2O$ (0.2), $ZnSO_4.7H_2O$ (0.1), $MnCl_2.4H_2O$ (0.03), $Na_2MoO_4.2H_2O$ (0.03), $NiCl_2.6H_2O$ (0.02), and $CuSO_4.5H_2O$ (0.01).

The pH of the solutions was adjusted to 7.0 with KOH (5.0 M). The solutions were then autoclaved separately and mixed aseptically to produce the medium. Samples were withdrawn at intervals of 2 hours for analyses of pH, cell concentration, and consumption of RCs.

ANALYTICAL METHODS

Cell growth was measured using a Marconi spectrophotometer, operated at a wavelength of 600 nm. The optical density values were correlated to the dry mass using a calibration curve. The determination of bacterial cell dry mass was performed by drying in an oven at 70 ºC to a constant weight, after filtration using a 0.22 μm membrane. The pH was monitored using a Marconi potentiometer. Measurements of RCs were performed according to the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1959). Glucose was used as a standard to produce the calibration curve.

The RCs were identified by high-performance liquid chromatography (Varian, Walnut Creek, CA, US), using a refractive index detector. A column suitable for organic acids was employed (Aminex HPX – 87H, 300 x 780 mm, Bio-Rad, Hercules, CA, US), maintained at 65 ºC (Klein and Leubolt, 1993). The mobile phase was an aqueous solution of H_2SO_4 (8.0 mM), and the flow rate was 0.6 mL/min. Retention times were determined using standard solutions of GalA, fructose, galactose, xylose, rhamnose and arabinose (Sigma), at concentrations in the range 0.2–10 g/L. The RCs' concentrations were expressed as a separate galacturonic acid group (GalA) and the sum of neutral sugars (NeutralS), compounds for fructose + xylose + galactose + rhamnose + arabinose.

RESULTS AND DISCUSSION

ACID HYDROLYSIS OPTIMIZATION

RCs' concentrations for different experiment conditionals were evaluated based on results of experimental design. The best experimental results obtained was 4.6 g/L of RCs, with 1.0% (v/v) sulfuric acid at 85 ºC, but the empirical model predict increase RCs' production with 1.0% (v/v) sulfuric acid at 100 ºC (Figure 1a). The Pareto chart (Figure 1c) and ANOVA (Table III) showed that all of the effects were significant ($p < 0.05$), and were therefore used in the prediction of an empirical model described by Equation 1. The parameter (1) L – Concentration of H_2SO_4 (% v/v) exerted the greatest influence on RC production, but this effect was negative, decreasing RC production with increasing of concentration of H_2SO_4. On the other hand, the parameters (2) L – Temperature (ºC) and Q – Temperature (ºC) exerted a positive influence on RC production. The distribution of the residuals (values predicted by the model *versus* observed values) showed that the deviations were normally distributed, and that there was a satisfactory correlation between the theoretical and experimental values (Figure 1b).

$$RC = 14.870 + 0.169*H_2SO_4 - 0.019*(H_2SO_4)^2 - 0.292*T + 0.002*T^2 - 0.005*H_2SO_4*T \quad (1)$$

Therefore, the formation of RCs was favored by decreasing the sulfuric acid concentration and increasing the temperature. Leitão et al. (1995), who used hydrochloric acid and trifluoroacetic acid to hydrolyze sunflower pectin, obtained similar results. These authors observed that the GalA yield increased at higher temperatures and lower acid concentrations. Garna et al. (2006) studied the hydrolysis of pectin using different concentrations of sulfuric acid and achieved the best results at acid concentrations around 1.0 M at 100 ºC and lower hydrolysis rates using acid concentrations

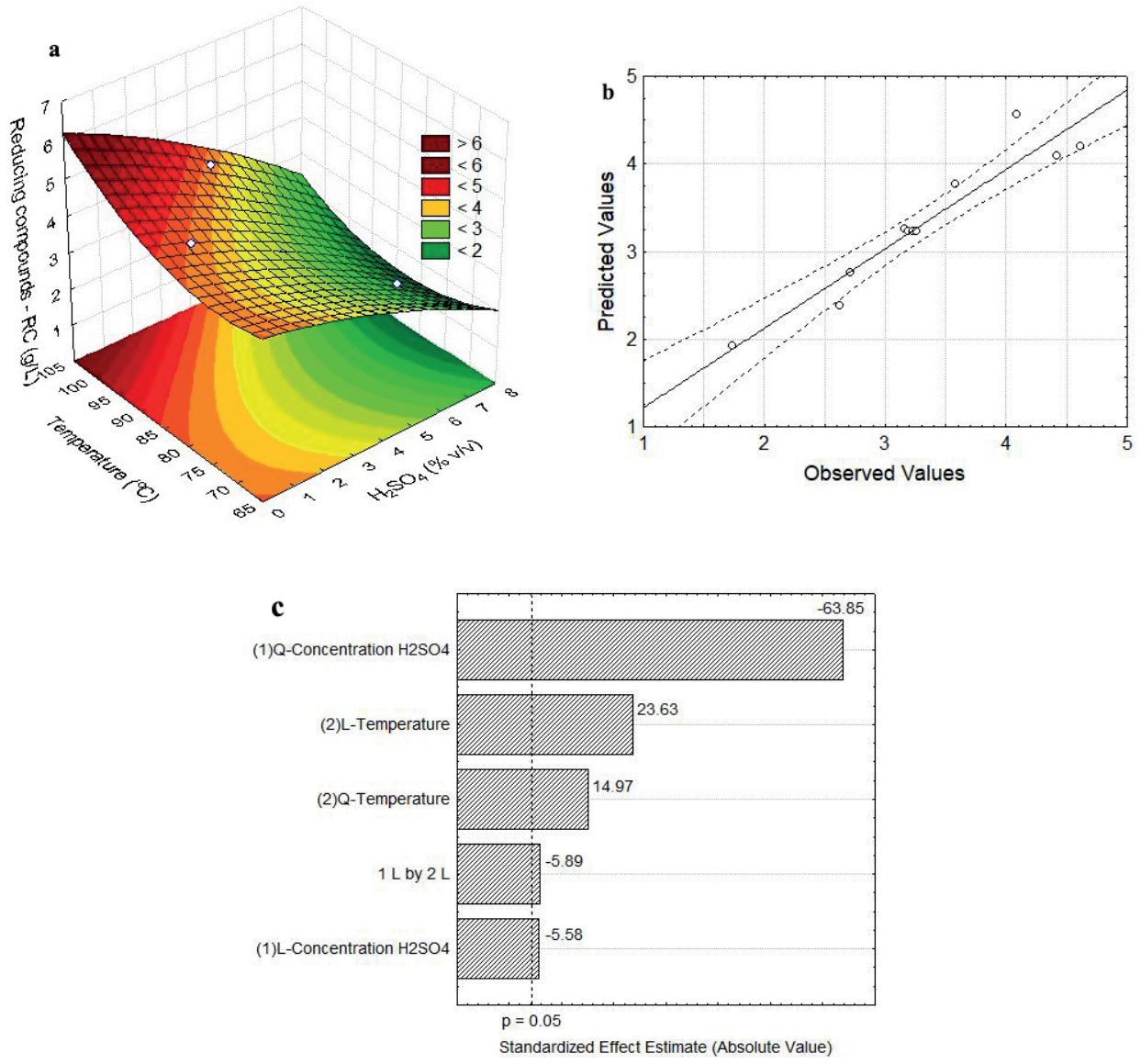

Figure 1 - Experimental design to pectin acid hydrolysis optimization – response surface (**a**), distribution of residuals (**b**), and Pareto chart (**c**) as a function of independent variables: temperature and H2SO4 concentration, and response variable: reducing compounds (RCs) production.

of 0.2 and 2.0 M. Other researchers also indicated the high stability of PGalA in acidic environments (Lim, et al. 2012, Min et al. 2011).

Garna et al. (2006) also observed the positive effect of temperature on the yield of free GalA. The positive influence of temperature on hydrolysis can be explained by greater solubilization of the polysaccharides at higher temperatures, and the greater resistance of the glycosidic bonds under milder hydrolysis conditions (Biermann 1988, De Ruiter et al. 1992). The negative effect of high acid concentrations can be explained by the decomposition of the RCs by other products. The combination of acid and high temperature may cause the formation of furfural derivatives, resulting in an imprecise determination of sugars (Wikiera

TABLE III
Acid hydrolysis optimization - ANOVA statistics data of independent variables and their interactions; dependent variable (RC concentration g/L).

Factor	SS	Df	MS	F	P
(1) L - Concentration of H_2SO_4 (% v/v)	5.149892	1	5.149892	4076.829	0.000245
(1) Q - Concentration of H_2SO_4 (% v/v)	0.039797	1	0.039797	31.505	0.030306
(2) L - Temperature (°C)	0.710374	1	0.710374	562.356	0.001774
(2) Q - Temperature (°C)	0.281626	1	0.281626	222.945	0.004455
1 L by 2 L	0.043972	1	0.043972	34.810	0.027546
Pure Error	0.002526	2	0.001263		
Total SS	6.976098	10			

et al. 2015). Such secondary reactions not only reduce the yield of the desired monosaccharides but also produce toxic compounds that prohibit the use of these hydrolysates in biological conversion processes.

The results clearly showed that optimum hydrolysis was achieved at higher temperatures and acid concentrations of up to 1.0% (v/v). Since it was not practically feasible to raise the temperature above 100 °C, the conditions chosen for the RC-release kinetics experiments were a temperature of 100 °C and an acid concentration of 1.0% (v/v) H_2SO_4.

ENZYMATIC HYDROLYSIS OPTIMIZATION

The best experimental results obtained was 8.8 g/L of RCs, with 11 UI/g of pectin and an agitation speed of 211.6 rpm, but the empirical model predict maximum concentration of RCs can be achieved using an enzyme concentration of 10.01 UI/g of pectin and an agitation speed of 230.3 rpm (Figure 2a). All of the effects were significant ($p < 0.05$) as showed the Pareto chart (Figure 2c) and ANOVA (Table IV), the observation that the second order effects were negative indicated that there was an optimum point for the two variables. Figure 2b shows that there was a good agreement between experimental data and numerical predictions. The codified model, optimized for the enzymatic hydrolysis of pectin after 24 hours of bioreaction, is given by Equation 2.

$$RC = -24.892 + 1.705*Enz - 0.067*Enz^2 + 0.219*Agit - 0.00044*Enz^2 \quad (2)$$

Thus, can conclude that both variables have a great influence on the enzymatic activity. Higher pectin concentrations increase the viscosity of the medium, agitation being indispensable to promote contact of the enzyme with the substrate. But at low concentrations such as that used in this work, high agitation speeds may hinder the formation of the enzyme-substrate complex. Songpim et al. (2010), working with pectate lyase enzyme, studied the effect of agitation speeds from 150 at 250 rpm and maximum response was obtained with 200 rpm, demonstrating the effect of this variable on the enzymatic activity.

In general, the enzyme activity increases as enzyme concentration increases. However, this increase is limited until the level of substrate saturation, as was demonstrated by Michizoe et al. (2001) when they studied the laccase concentration effect from 0 at 15 μM over degradation of *o*-chlorophenol. The authors observed that after 11 μM, the degradation rate was practically stable.

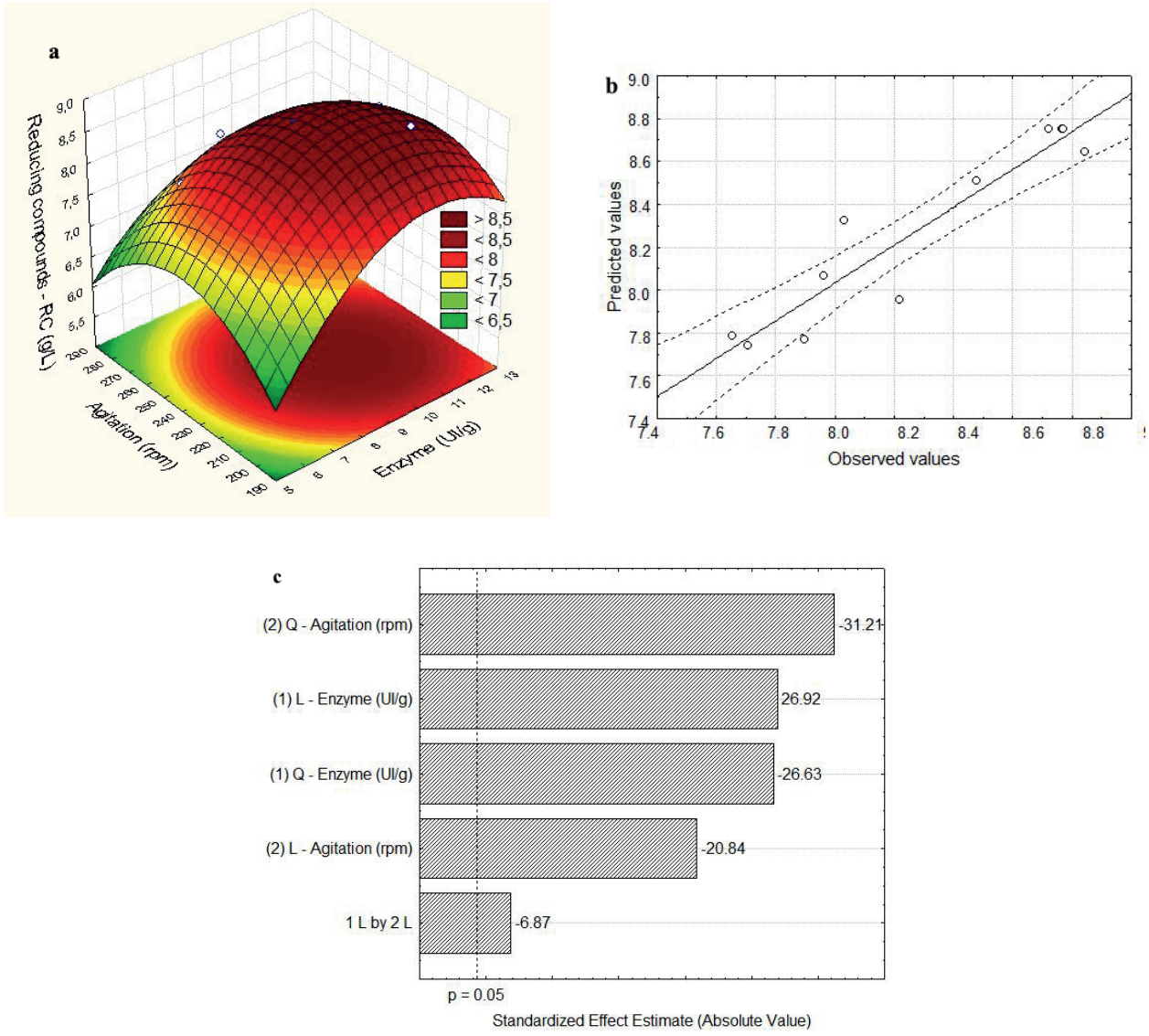

Figure 2 - Experimental design to pectin enzymatic hydrolysis optimization - response surface (**a**), distribution of residuals (**b**), and Pareto chart (**c**) as a function of independent variables: agitation speed and enzyme concentration, and response variable: reducing compounds (RCs) production.

COMPARISON BETWEEN KINETICS OF ACID AND ENZYMATIC HYDROLYSIS

The kinetics during at acid hydrolysis (Figure 3a) revealed a higher rate of hydrolysis during the first 15 minutes, with a gradual increase up to 4 hours, when a maximum concentration of 6.0 g/L was achieved (which was higher than the 5.3 g/L predicted by the model). Then there was a slight decline until the end of the 5-hour hydrolysis period. The release profiles of the carbohydrate groups were in agreement with the results obtained for RCs, with the concentration of GalA (maximum 3.2 g/L reached in a 4-hour hydrolysis period) exceeding that of NeutralS (maximum concentration 1.8 g/L reached in a 1-hour hydrolysis period).

Pectins with a high degree of esterification are commonly extracted using hot water (60 at 100 °C) at pH ranges from 1.5 to 3.0 for several hours

TABLE IV
Enzymatic hydrolysis optimization - ANOVA statistics data of independent variables and their interactions; dependent variable (RC concentration g/L).

Factor	SS	Df	MS	F	P
(1) L - Enzyme (UI/g)	0.524779	1	0.524779	724.5920	0.001377
(1) Q - Enzyme (UI/g)	0.513524	1	0.513524	709.0513	0.001407
(2) L - Agitation (rpm)	0.314697	1	0.314697	434.5194	0.002293
(2) Q – Agitation (rpm)	0.705515	1	0.705515	974.1444	0.001025
1 L by 2 L	0.034157	1	0.034157	47.1628	0.020552
Pure Error	0.001448	2	0.000724		
Total SS	2.061697	10			

(Koubala et al. 2008). These extraction conditions of pectin could explain the elevated initial rate of free GalA. The resistance to acid hydrolysis of the glycosidic linkages is variable and runs as follows: GalA---GalA > GalA---Rha > Rha---GalA > sugar neutral–sugar neutral, GalA monomers being the last to be released (Novoselskaya et al. 2000). This could explain the gradual release of RCs up to the end of the hydrolysis process. The lower resistance of the glycosidic linkages between neutral sugars are perceived in the behavior curve of the NeutralS that have a higher rate of release in the first 30 min of hydrolysis.

These results are similar to those obtained in earlier studies by Garna et al. (2006), who hydrolysed a highly esterified pectin using different concentrations of sulfuric acid at 100 ºC. For all treatments, a gradual release of GalA was observed during the first hours of the process, a decline in the concentration of free GalA being observed after different times for each treatment. According to the authors, the continuation of hydrolysis conditions for long periods led to rates of destruction of free GalA that exceeded the release rates.

In addition to the GalA monomers, the pentoses present in pectin can be degraded to formation of furfural derivatives, which makes the precise determination of sugars impossible (Wikiera et al. 2015, Medina et al. 1942). The results indicated that formation of these degradation products was low for up to 4 hours of hydrolysis under the conditions employed, so that the hydrolysate could therefore be used in the culture medium.

In order to confirm the results obtained in enzymatic hydrolysis optimization, it was performed an experiment in triplicate using the optimized conditions achieved previously. The RC release kinetics was monitored during 24 hours of enzymatic hydrolysis (Figure 3b). The rate of hydrolysis was faster during the first 30 minutes and remained high for 8 hours. This was succeeded by a gradual hydrolysis for the remaining period of 24 hours, and at the end the RC concentration was 9.34 ± 0.16 g/L (which was greater than the value of 8.85 g/L predicted by the model).

The release profiles of the GalA and NeutralS groups were broadly similar to that of the RCs. There was a greater initial release of the GalA during the first hour of hydrolysis, followed by gradual release for the remaining period of 24 hours and reaching a maximum concentration of 8.4 g/L. The NeutralS group showed a gradual release up to 19 hours, after which the concentration remained constant up to 24 hours of hydrolysis (maximum concentration 1.4 g/L).

Bélafi-Bakó et al. (2007) also observed a decrease in the RC and GalA release rate during the course of the hydrolysis process. Those authors

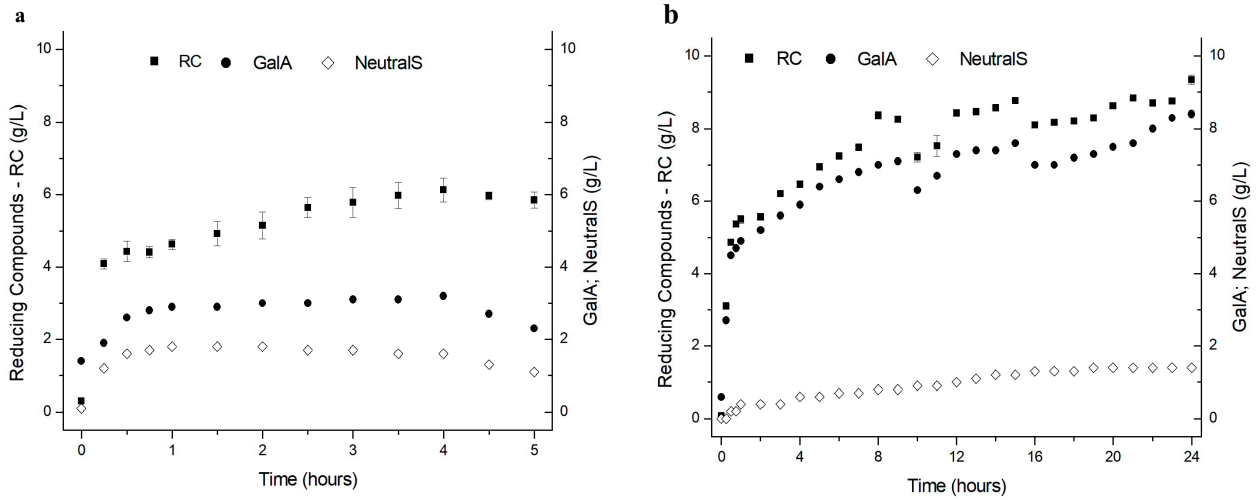

Figure 3 – Release kinetics of reducing compounds during acid hydrolysis of pectin (**a**) – with 1.0% v/v H2SO4; 100 oC; 1.0% (w/v) pectin; and during enzymatic hydrolysis of pectin (**b**) - with 10.01 UI/g of enzyme; 230.3 rpm; 1.0% (w/v) of pectin.

utilized different initial concentrations of free GalA and demonstrated that the product of hydrolysis inhibited the enzymatic activity. The neutral sugars, found at lower concentrations in pectin, are probably depolymerized due to the lower resistance of their glycosidic bonds (Novoselskaya et al. 2000), with an acidity of pH 4.0 and a temperature of 50 ^{o}C being sufficient to cause their release.

Given an initial pectin concentration of 1.0% (w/v), the average yields of the enzymatic and acid hydrolyses were 93.0% and 60.0%, respectively. The enzymatic method was therefore more efficient for the production of RCs from the hydrolysis of pectin.

CELL GROWTH

The hydrolysates obtained by both methods were diluted for use in the culture medium formulations. Initial concentrations of RCs were different because it was considered the initial substrate concentration in the hydrolysis process (pectin 1.0% w/v). Therefore, it achieved 3.82 and 5.30 g/L RCs in the initial media formulated with the hydrolyzed acid and enzymatic, respectively. The growth of *C. necator* and parameters kinetics of process were followed as a function of time. Cell concentration, pH, and substrate consumption are shown in Figures 4 and 5.

When the chemical hydrolysate was used (Figure 4a), growth of the microorganism began immediately after cell inoculation and continued for around 10 hours, with μ_{Max} of 0.26 h^{-1}. For the culture using the enzymatic hydrolysate (Figure 5a), there was an adaptive phase lasting for around 2 hours, followed by exponential growth up to 12 hours, with μ_{Max} of 0.29 h^{-1}. The results can be compared with those obtained by other authors (Table V).

The pH remained at around 7.0 throughout the culture period, using both formulations. The consumption of RCs was related to the growth phase, with residual values of 2.8 and 3.7 g/L for media formulated with the hydrolyzed, acid and enzymatic, respectively (Figure 4b and 5b). We see a residual amount of RCs that can't be metabolized by *C. necator*, which was also observed by other authors using other carbon sources (Baei et al. 2009, 2011, Locatelli et al. 2011, Lagunes and Winterburn 2016).

This RC residual in both hydrolysates could be related to the presence of oligomers with reducing terminal residues that the microorganism

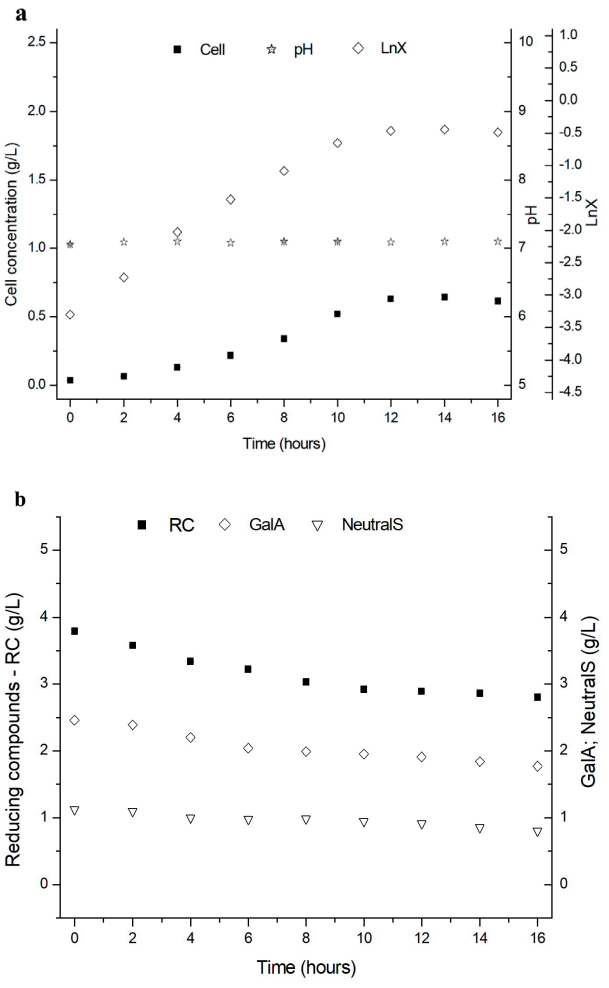

Figure 4 – Cell growth and pH (**a**); and consumption of substrates (**b**), during culture of *C. necator* in mineral media containing chemical pectin hydrolysate.

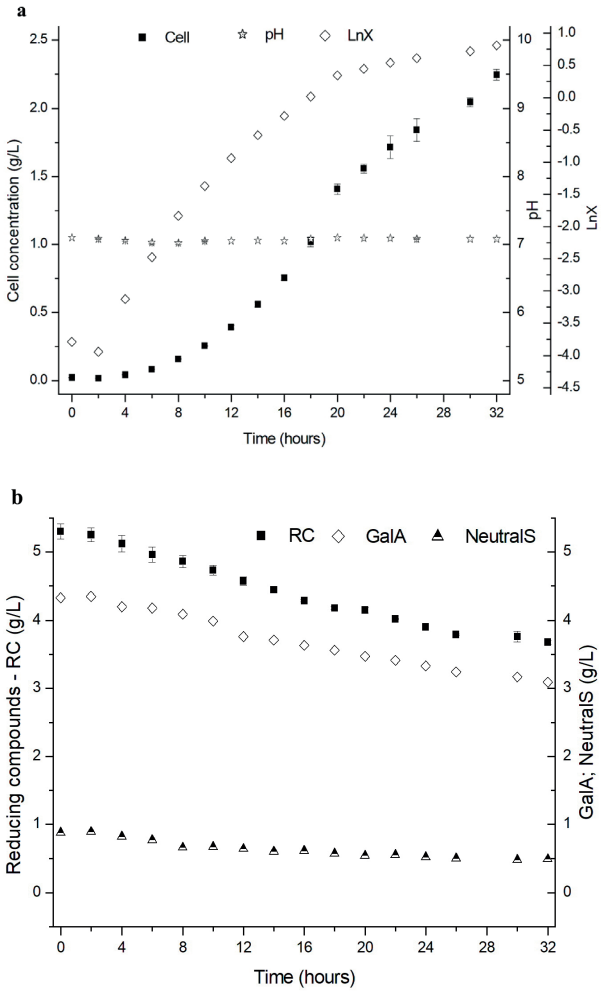

Figure 5 - Cell growth and pH (**a**); and consumption of substrates (**b**), during culture of *C. necator* in mineral media containing enzymatic pectin hydrolysate.

is unable to metabolize. The furanic aldehydes formed by degradation of GalA and sugar affect the microorganism's metabolism, being toxic for fungus (Szengyel and Zacchi 2000), yeasts (Taherzadeh et al. 1999) and bacteria (Zaldivar et al. 1999) and undesirables in culture medium formulations, which could explain the low final cellular concentration using the medium formulated with the hydrolyzed acid.

CONCLUSIONS

The production of RCs from the hydrolysis of pectin was more efficient using an enzymatic method, with a yield 33.0% higher than that achieved using acid hydrolysis. The release profiles of the GalA and NeutralS groups were broadly similar to that of the RCs, to both hydrolysis processes. Moreover, it proves that drastic conditions as the high acid' concentration can be negative over RC' production.

The mineral medium formulation containing an enzymatic hydrolysate provided a higher final cell concentration during growth of *C. necator*, with a specific growth rate that was superior to that obtained using a chemical hydrolysate. In this way, enzymatic hydrolysis can be used in the saccharification of agroindustrial waste pectin,

TABLE V
Cell growth rates of *Cupriavidus necator* using Chemical and Enzymatic Hydrolysate, compared with other carbon sources.

References	Microbial Strain	Carbon source Type	Initial Carbon concentration ($g\ L^{-1}$)	$Y_{x/s}$	μ_{Max}
This paper	***C. necator*-DSM 545**	**Chemical hydrolysate**	**3.82 (in reducing compounds)**	**0.58**	**0.26 h^{-1}**
		Enzymatic hydrolysate	**5.30 (in reducing compounds)**	**0.62**	**0.29 h^{-1}**
Yousuf and Winterburn 2016	*C. necator* H16	Date seeds extract	10.80 (of fructose)	0.68	0.13 h^{-1}
Lagunes and Winterburn 2016	*C. necator* H16-DSM 428	Orange juicing waste	14.94 (of fructose)	0.40	0.179 h^{-1}
Aramvash et al. 2015	*C. necator*-ATCC 17699	Arabinose Glucose Fructose Sucrose	20.00 (of each)	---	---
Figueiredo et al. 2014	*C. necator*-IPT 026 *C. necator*-IPT 027 *C. necator*-IPT 028	Residual Glycerin from Biosiesel Glucose	30.00 (of each)	---	---
Locatelli et al. 2011	*C. necator*-DSM 545	Galacturonic acid	15.00	---	---
		Pectin hydrolysate	3.82 (in reducing compounds)	0.55	0.26 h^{-1}
Baei et al. 2011	*C. necator*-DSM 545	Glucose	40.00 (of each)	0.53	0.17 h^{-1}
		Fructose		0.50	0.125 h^{-1}
		Sugarcane Molasses (Inverted sugar)		0.55	0.42 h^{-1}
Dalcanton et al. 2010	*C. necator*-DSM 545	Rice starch hydrolysate	30.00 (in reducing sugars)	---	0.238 h^{-1}
Cavalheiro et al. 2009	*C. necator*-DSM 545	Waste glycerol (GRP)	---	0.45	0.15 h^{-1}
		Commercial Glycerol (PG)		0.37	0.12 h^{-1}
Marangoni et al. 2001	*R. eutropha*-DSM 545 (Nowdays *C. necator*)	Inverted sugar	20.00 (of each)	---	0.26 h^{-1}
		Glucose			0.23 h^{-1}
		Fructose			0.21 h^{-1}
		Galactose			0.13 h^{-1}

Source: The authors.

and the hydrolysis product can be applied in the process of *C. necator* growth, with potential for SCP production or for PHA production.

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AUTHOR CONTRIBUTIONS

All authors conceived and planned the experiments. G.O. Locatelli carried out the experiments. L Finkler and C.L.L Finkler contributed to samples preparation. All authors contributed to the interpretation of the results. G.O. Locatelli took the lead in writing the manuscript. All authors provided critical feedback and contributed to the final version of the manuscript.

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