Enzymatic hydrolysis of carbohydrates in by-products of processed rice

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ABSTRACT: Over post-harvest steps of rice, from pre-cleaning to processing, a large amount of by-product is generated. Some of these by-products, due to their high starch and fiber content can be used in ethanol production. The objective was to evaluate the effect of enzymatic hydrolysis conditions on the production of reducing sugars, from pre-cleaning residue and type III paddy rice, as well as the effect of the pre-treatment of its fibers, targeting the use of these residues in ethanol fuel production. The proximate analysis was performed, followed by the pre-treatment of samples. Enzymatic hydrolysis was conducted in two ways: using one enzyme at a time or applying them simultaneously. The starch content was 41.18 and 53.41%; the fibers were 30.44 and 23.39%, of which 6.53 and 4.41% were lignin, for the pre-cleaning residue and paddy rice, respectively. Alkaline pre-treatment reduce lignin content by 47.94 and 18.23% for the pre-cleaning residue and type III paddy rice, respectively. Hydrolysis efficiency was 22.61 and 15.32% for the cellulase enzyme, and 82.18 and 87.07% for the amylolytic enzymes in the pre-cleaning residue and type III paddy rice, respectively. The hydrolysis with the separated enzymes presented higher reducing sugar yields. Therefore, the pre-cleaning residue and type III paddy rice can be used for ethanol production by its enzymatic hydrolysis, aiming to add value and to increase the sustainability of the rice production chain.

Key words: enzyme, Oryza sativa, reducing sugar, residue, starch.

INTRODUCTION

Oryza sativa (rice) is one of the most important cereals for the food chain. Brazil is the 9th largest producer in the world and the largest in Latin America, having produced 11.4 million tons during the 2018 harvest (FAO, 2018; IBGE, 2019).

The grains are composed of proteins, lipids, fibers, ashes, minerals, vitamins and starch. The latter represents the largest amount in the grain composition (WALTER, et al., 2008; ZIEGLER et al., 2017). Factors such as cultivar, soil preparation, seed quality, climate, storage conditions and harvest time can influence the composition, yield and quality of the grain (KAMINSKI et al., 2013; ZIEGLER et al., 2017).

Before it is marketed, polished rice undergoes pre-cleaning, drying, storage, classification and processing operations inside a processing plant. During the pre-cleaning phase, impurities such as straw, husks, broken grains, stones and other materials are removed. Impurities can slow down
the drying process, accelerate the development of microorganisms, and facilitate insects’ proliferation (HALBERSTADT et al., 2015). From field to market, about 2 million tons of by-products are generated annually in Brazil (IPEA, 2012). Some of them are currently used for animal feed or energy production.

Domestic and International markets have requirements for paddy or processed grain, as well as for its fragments. For the Brazilian market, the Normative Instruction number 6 of 2009, from the Ministry of Agriculture, Livestock and Supply (BRASIL, 2009) defines classes based on the grain quality. Rice is classified according to the occurrence of defects, from type I to non-compliant. Lower quality rice has a reduced market value, which may turn its processing unfeasible.

Thus, by-products or low-quality grains can become a co-product, considering the development of technologies that can use it alternatively, adding value to the production chain (NASCIMENTO FILHO & FRANCO, 2015).

The transformation of by-products from agricultural raw materials processing has been widely studied, emphasizing biofuel production, that can enhance profits of the production chains and its sustainability, not to mention that the by-products may not compete with food production (NONES et al., 2017; TAKANO & HOSHINO, 2018). Brazil stands out as one of the largest ethanol producers in the world, having produced approximately 33.14 billion liters in the 2018/2019 season (55.7% of global production). However, sugar cane remains the country’s main raw material (CONAB, 2019), which can create a critical dependence on this input.

Low-quality paddy rice and the by-product gathered from the pre-cleaning can be considered for ethanol production, after its hydrolysis that allows the formation of reducing sugar and the subsequent alcoholic fermentation (RAUL et al., 2016). The high content of starch and fiber of those by-products can render their transformation into fermentable sugars technically and economically feasible.

The enzymatic hydrolysis of starch and fibers results in higher conversion into sugar than the acid hydrolysis process (FERREIRA et al., 2013). The selective action of the enzyme on the molecule is the key factor for this result. Cellulase acts selectively on the fiber, while amylloglucosidase and alpha-amylase act only on starch, under specific conditions of pH, temperature, concentration and reaction time; reducing the formation of secondary products (BISSWANGER, 2014).

Starch is composed of two polysaccharides: amylose and amylopectin and its hydrolysis produce oligosaccharides, dextrins and glucose (ECKERT et al., 2018). For fibers, cellulose hydrolysis generates glucose and celllobiose, and hemicellulose degradation produces arabinose, xylose, mannose, rhamnose, galactose and glucose (BALAT, 2011).

The objective was to evaluate the technical feasibility of the use of pre-cleaning residue and paddy rice type III as raw materials for ethanol production from enzymatic hydrolysis.

MATERIALS AND METHODS

Sample preparation

The pre-cleaning residue (PCR) and the paddy rice classified as type III (PRT3) were obtained from a rice processing plant located in Lagoa da Confusão, State of Tocantins, Brazil. Pampeira and IRGA 425 cultivars comprised the samples.

Samples were dried in an oven operating by forced circulation of air (Model SL-102, Solab) at 60 °C for 48 h (INSTITUTO ADOLFO LUTZ, 2018). After drying, samples were crushed and sieved at 1 mm in a knife mill (Model MA 340, Marconi). The prepared material was stored in sealed containers prior to being analyzed and hydrolyzed.

Proximate analysis

The proximate analysis was performed for both by-products (PCR and PRT3) according to the AOAC international standards (AOAC, 2000), for the following parameters: Moisture (AOAC 934.01), ash (AOAC 924.05), crude fat (AOAC 920.39C) and crude protein (AOAC 920.87), using the conversion factor of 6.25. Total fiber and lignin content were determined by the dietary fiber method (VAN SOEST et al., 1991) and the starch content by the Fehling method (AOAC, 2016). Cellulose concentration was estimated using the difference of total fiber, lignin content and the neutral detergent fibers - NDF.

The non-fibrous carbohydrate content was estimated by equation 1 (SNIFFEN et al., 1992).

\[
NFC = 100 - (CP + CF + ASH + TF)
\]

where:

NFC = Non-Fibrous Carbohydrates (%);
CP = Crude protein (%);
CF = Crude Fat (%);
ASH = Ash (%);
TF = Total fibres (%).

Pre-treatment of samples

Once both by-products were characterized, a part of the samples of PCR and PRT3 were submitted to the pre-treatment to break its fibers. It aimed the
hydrolysis of the lignocellulosic complex for the exposure of the cellulose molecules to the action of the specific enzymes. To do so, 10 g of sample was added in 100 mL of 0.1 M NaOH solution and then autoclaved (vertical AV analog model, Phoenix) for 1 h at 121 °C. The samples were taken out from the autoclave and left to stand for 24 h at room temperature. The material was filtered and the solid portion retained in the filter was washed by distilled water until reaching pH 7.6 at 26.1 °C. Washed, the solid material was dried in an oven operating by forced circulation of air (Model SL-102, Solab) at 80 °C for 30 h (SUKUMARAN et al., 2009).

After pre-treatment, lignin (VAN SOEST et al., 1991) and starch contents were determined based on the Fehling method (AOAC, 2016) in order to evaluate the pre-treatment efficiency for lignin removal.

The remaining part of the samples, which were not submitted to pre-treatment, was taken to enzymatic hydrolysis and the consequent production of reducing sugars by the action of enzymes: alpha-amylase (StarMax 300®), amyloglucosidase (StarMax GA 300®) and cellulase (CeluMax C®), produced and marketed by PROZYN®. As the environmental and the medium conditions, such as pH, temperature (T) and concentration (E) strongly influence the efficiency of the enzymatic process, they were set during a pre-test stage.

**Enzymatic hydrolysis**

Once ideal values for pH, temperature and concentration were determined, the samples were submitted to hydrolysis. The three enzymes were tested, individually, to set the optimal time of reaction. Yields were determined at 24 h, 48 h and 72 h from the beginning of reaction for cellulase and amyloglucosidase and after 90, 120 and 150 minutes for alpha-amylase. The range for reaction times and for the chemical-physical characteristics of the medium, were set based on the guidelines from the enzyme technical sheet.

The part of the samples that were hydrolyzed by the action of each enzyme at a time were submitted to the following procedure: 20 g of untreated sample was added to 0.4% of cellulase diluted in 50 mL of sodium acetate buffer solution. The pH of buffer solution was stabilized in 5.0. The mixture of the solution, cellulase and sample was placed inside a water bath and stirred for 48 h at 50 °C. After cellulase acted over samples, 50 mL of sodium acetate buffer solution (pH 4.5) and 0.1% of amyloglucosidase was added to the mixture. It stirred again this mixture in a water bath for 24 h at 58 °C. Finally, the mixture received 0.3% of the alpha-amylase diluted in 50 mL of phosphate buffer solution (pH 6.0) and it was stirred in the water bath at 90 °C, for 90 minutes.

As each enzyme is highly specific and its optimal performance can occur at conditions close to those for another enzyme, the reducing sugar yield was also tested for cellulase and amyloglucosidase acting simultaneously, in order to reduce the total reaction time, optimizing the process.

For the analysis of the simultaneous activity of the cellulase and amyloglucosidase followed by the alpha-amylase action, 0.4% of cellulase and 0.1% of amyloglucosidase were diluted in 50 mL of sodium acetate buffer solution (pH 4.5) which received 20 g of no-pretreated sample. This mixture was stirred in a water bath for 48 h at 60 °C followed by the addition of 0.3% of alpha-amylase enzyme diluted in 50 mL of phosphate buffer solution (pH 6.0), which was stirred in a water bath at 90 °C for 90 minutes.

After each reaction time for the individual or simultaneous action of enzymes, 2 mL of hydrolysate was taken out. 2 mL of NaOH 0.05 M was added to hydrolysate for enzyme inactivation. This inactivated hydrolysate was centrifuged (Model 206, Fanem) at 3600 rpm for 10 min and the supernatant was used to determine the reducing sugars content by 3.5-dinitrosalicylic acid - DNS method (MILLER, 1959).

The efficiency of the hydrolysis process for amyloglucosidase and alpha-amylase was calculated by equation 2 (KOWALSKI et al., 2017) and for cellulase by equation 3 (ANDRADE et al., 2019):

\[
\eta = \frac{[\text{Glu}]}{[\text{Stc}]} \times 100 
\]

where:

\( \eta \): hydrolysis yield (%);

\([\text{Glu}]\): glucose concentration (%);

\([\text{Stc}]\): starch concentration (%);

fs: conversion factor for starch (1.11).

\[
\eta = \frac{[\text{Glu}]}{[\text{Cel}]} \times 100 
\]

where:

\( \eta \): hydrolysis yield (%);

\([\text{Glu}]\): glucose concentration (%);

\([\text{Cel}]\): cellulose concentration (%);

fc: conversion factor for cellulose (0.9).

The total reducing sugar concentration obtained after the hydrolysis of fiber and starch was analyzed for each fraction: arabinose, cellobiose,
galactose, glucose, mannose, xylose and sucrose. The individual sugar concentrations were determined by high-performance liquid chromatography (HPLC) using a refractive index (RI) detector, according to AOAC 977.20 (AOAC, 2016).

**Statistical analysis**

The data from proximate analysis were submitted to descriptive statistics. Other data were submitted to the normality test followed by ANOVA. The means were compared by the Tukey’s test. All statistical procedures were performed by Action Stat Pro software (ESTATCAMP, 2017).

**RESULTS**

The proximate analysis of PCR revealed that 48.60% of the material are non-fibrous carbohydrates; 30.44 ± 0.90% represented total fibers, being 11.50% cellulose; 9.63 ± 0.23% of crude protein; 5.92 ± 0.36% of crude fat; 5.91 ± 0.25% of moisture and 5.41 ± 0.39% of ash.

The results for PRT3 shows 59.73% of non-fibrous carbohydrates; 23.39 ± 0.86% of total fibers, of which 8.34% are cellulose; 7.40 ± 0.13% of moisture; 7.18 ± 0.49% of crude protein; 6.56 ± 0.27% of crude fat and 3.14 ± 0.11% of ash.

The alkaline pre-treatment reduced 47.94% of the lignin content of PCR samples and 18.60% of PRT3. But 77.76 and 75.55% of starch were also removed from PCR and PRT3, respectively, by the alkaline pre-treatment (Table 1).

The optimal values of pH, temperature (T) and concentration (E) for enzymatic hydrolysis using cellulase, amyloglucosidase and alpha-amylase did not differ among PCR and PRT3 (Table 2).

The time of exposure to some enzymes influenced the conversion rate of carbohydrates into reducing sugar for both by-products. The influence of time was observed for cellulase and for alpha-amylase, not for amyloglucosidase. For cellulase, the best results for reducing sugar yields were achieved at a time of reaction of 48h. For alpha-amylase, the optimal time was 90 min, and 24h for amyloglucosidase (Table 3).

Amyloglucosidase was responsible for most of the reducing sugar produced during the hydrolysis using one enzyme at a time, followed by alpha-amylase and cellulase, likewise for the hydrolysis efficiency. The yields calculated for the simultaneous action of amyloglucosidase and cellulase were lower than the results of its isolated action (Table 4).

The concentration of total reducing sugar was higher for the hydrolysis by the separate enzymes (Figure 1) and glucose was the only sugar retrieved from the enzymatic hydrolysis of starch and fiber from PCR and PRT3 (Table 5).

**DISCUSSION**

The starch content determines the feasibility of a raw material to get good yields from its hydrolysis and subsequent fermentation for ethanol production. Concentrations of 41.18% in PCR and 53.41% in PRT3 qualifies these by-products for ethanol production, since other traditionally used raw materials such as maize, sorghum and cassava have 67, 70 and 85% starch, respectively (HILL, et al., 2012; SOARES et al., 2017; URBANO et al., 2017). Further, ethanol production may be greater if fibers can be converted into reducing sugars too, after hydrolysis of these polysaccharides (RAELE et al., 2014).

The proximate analysis of PCR shows similar results found for other rice strains, grown in different Brazilian regions (KAMINSKI et al., 2013; AMAGLIANI et al., 2017). Therefore, these results

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Table 1 - Lignin and starch contents (Mean ± SD g/100g) for pre-cleaning residue (PCR) and for type III paddy rice (PRT3) before and after pre-treatment.

| Compounds | PCR Before | PCR After | PRT3 Before | PRT3 After |
|-----------|------------|-----------|-------------|------------|
| Lignin    | 6.53 ± 0.45 a | 3.40 ± 0.24 b | 4.41 ± 0.18 A | 3.59 ± 0.22 B |
| Starch    | 41.18 ± 0.69 a | 9.16 ± 0.32 b | 53.41 ± 1.04 A | 13.06 ± 0.39 B |

*Means followed by the same letters in the line do not differ by the F test at p < 0.05.
can be replicated for other producing regions in Brazil or perhaps in the world. However, it must be considered that there are many materials that are part of PCR and it can vary among different processing plants, such as leaves, bark, soil, other parts of the plant and so on. Therefore, its chemical composition may be different depending on the cultivar, harvest time, weather conditions or processing procedures.

The reduction in lignin content via alkaline pre-treatment indicates the rupture of the lignocellulosic complex (KIM, et al., 2016). Similar results for delignification of rice husk and straw were presented by other authors (AZEVEDO et al., 2016, TAKANO & HOSHINO, 2018). The highest reduction for lignin content in PCR samples is due to the presence of layers of silica, constituting a kind of shield that prevents the action of alkali (MARIN et al., 2015). Conversely, the pre-treatment caused an undesired effect, by reducing the starch content once hydrolyzed with alkali. It minimizes the yield of reducing sugar, so the use of pre-treatment was not recommended for the tested by-products.

Cellulase is an enzymatic complex that acts in synergism, so the hydrolysis process required a longer reaction time. However, if glucose and celllobiose formed by hydrolysis remained in the same medium for a long time, they inhibited the action of cellulase, decreasing the reducing sugar concentrations (SINGH, et al., 2014).

Hydrolysis by amyloglucosidase can occur for long periods, since it acts on the non-reducing end, breaking both connections: α-1,4 and α-1,6 (TORRES, et al., 2012). Conversely, alpha-amylases hydrolyze starch faster, as these enzymes only break the α-1,4 bonds along the amylase and amylopectin chains (TORRES et al., 2012). Most enzymes produced by bacteria are stable at 90°C, but they lose stability over time, which may decrease their activity (KAHALLA et al., 2014).

A higher relation between starch and fibers concentrations, and the occurrence of lignin that hinders enzymatic access to cellulose (CASTRO et al., 2017), explains the higher conversion rate into reducing sugar by the action of amylolytic enzymes (TORRES et al., 2012).

The higher efficiency of amyloglucosidase can be justified by the high concentration of amylopectin compared to amylose in rice (DENARDIN et al., 2012). However, the lower reducing sugar yield for simultaneous hydrolysis is due to the pH and the temperature of the medium. They were adjusted to an intermediary condition for both enzymes, as close as possible to the optimal values, but it may reduce enzymatic activity (BISSWANGER, 2014).

| Enzyme     | pH | T (°C) | E (%) |
|------------|----|--------|-------|
| Cellulase  | 5.0| 50.0   | 0.4   |
| Amyloglucosidase | 4.5| 58.0 | 0.1   |
| Alpha-amyrase | 6.0| 90.0  | 0.3   |

| Enzyme           | Reaction time | PCR       | PRT3    |
|------------------|---------------|-----------|---------|
|                  | 90 min | 120 min | 150 min | 24 h | 48 h | 72 h |
| Cellulase        | N.A.    | N.A.    | N.A.    | 2.02±0.53 b | 3.51±0.48 a | 2.33±0.19 b |
| Amyloglucosidase | N.A.    | N.A.    | 37.37±1.50 a | 37.19±0.23 a | 36.87±0.76 a |
| Alpha-amyrase    | 17.51±0.48 a | 16.33±0.19 b | 16.037±0.26 b | N.A. | N.A. |
|                  | N.A.    | N.A.    | N.A.    | 2.74±0.17 b | 3.78±0.08 a | 2.71±0.10 b |
| Amyloglucosidase | N.A.    | N.A.    | 25.50±0.41 a | 25.28±0.21 a | 25.09±0.12 a |
| Alpha-amyrase    | 12.70±0.52 a | 11.24±0.18 b | 11.07±0.27 b | N.A. | N.A. |

*Means followed by the same letters in the line do not differ by the Tukey's test at p < 0.05.
**NA - Does not apply.
sugar yield was lower due to the low concentration of cellulose in the tested by-products, that results in the dispensable use of this enzyme for ethanol production using the rice by-products.

The absence of arabinose, galactose, xylose and mannose in sugar analysis demonstrated that hemicellulose was not degraded (Balat, 2011). Since the action of enzymes is specific, the environmental and the medium conditions were not sufficient for the hydrolysis of the hemicellulose.

The cellobiose formed by cellulose hydrolysis was degraded into glucose by the action

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**Table 4** - Reducing sugar (SC) and efficiency (η) for hydrolysis by cellulase, amyloglucosidase, alpha-amylase, and simultaneously by cellulose plus amyloglucosidase.

| Enzymes         | SC (%)       | η (%) | SC (%) | η (%) |
|-----------------|--------------|-------|--------|-------|
| Cellulase       | 2.89 ± 0.21  | 22.61 | 1.42 ± 0.23 | 15.32 |
| Amyloglucosidase| 25.49 ± 2.06 | 56.30 | 35.28 ± 3.44 | 59.67 |
| Alpha-amylase   | 11.72 ± 2.89 | 25.88 | 16.26 ± 6.43 | 27.40 |
| Amyl + Cellulase| 21.73 ± 1.27 | N.A.  | 25.11 ± 1.08 | N.A.  |

NA - Not applicable (reducing sugar content comes from starch and cellulose).

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**Figure 1** - Reducing sugar concentration (Mean ± SD) after enzymatic hydrolysis of PRT3 (A) and PCR (B) by the action of enzymes separately or simultaneously. Means followed by the same letter do not differ statistically by the F test at p < 0.05.
Table 5 - Sugars concentration of hydrolyzed samples, determined by HPLC-RI.

| Sugars     | PCR (g/100g) | PRT3 (g/100g) |
|------------|--------------|---------------|
| Arabinose  | ND           | ND            |
| Cellobiose | ND           | ND            |
| Galactose  | ND           | ND            |
| Glucose    | 36.25        | 49.26         |
| Manose     | ND           | ND            |
| Xylose     | ND           | ND            |
| Sucrose    | ND           | ND            |

ND - Not Detected.

of β-glucosidase that compose the enzymatic complex (ODEGA & PETRI, 2010). The action of amylolitic and cellulolytic enzymes on the material was confirmed by the exclusive presence of glucose in the hydrolysate, which may increase the yield of alcoholic fermentation by *Saccharomyces sp.* yeasts.

CONCLUSION

Alkaline pre-treatment was effective for lignin reduction but caused the loss of a part of the starch in by-products. Therefore, the use of non-pretreated by-products ensures higher sugar concentrations for ethanol production.

Enzymatic hydrolysis using one enzyme at a time presented a higher yield in reducing sugars. Acting simultaneously, the enzymes had its action influenced by the pH and the temperature. Optimum temperature, pH, concentration and time of reaction can increase the performance of enzymatic hydrolysis of rice by-products.

The use of amyloglucosidase and alpha-amylase to hydrolyze pre-cleaning residue and low-quality paddy rice can make feasible the use of these by-products for ethanol production. As a result, increasing the diversity of sustainable raw materials can supply this fuel chain.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS’ CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors revised the manuscript and approved the final version.

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