

# Yeasts from rumen of adult Zebu cows and fermentations of lignocellulosic materials

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## Research article

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# Abstract

## Background

Commercial yeast strains are a promising alternative to chemical additives in ruminants; but are not self to the rumen ecosystem. In this study, the aim was to identify and evaluate the growth, mass production, and enzymatic activity of yeast strains naturally isolated from bovine rumen in submerged fermentations containing roughages with high concentration of lignocellulose. An experiment was initially conducted to compare the degradation of *Urochloa decumbens* (UD) hay or sugarcane bagasse (SB) by eight yeast isolates and a control without microorganisms. Subsequently, further experiments were performed to compare the degradation of the two substrates by two yeast strains (V5 and V62) selected (from the eight used in the initial screen) and a control without microorganisms for four different fermentation periods.

## Results

Among the identified strains, *Pichia kudriavzevii* was the most common, followed by *Rhodotorula mucilaginosa* and *Candida tropicalis*. The strain (V5 *R. mucilaginosa*) showed higher mass production than other strains of this specie in fermentations with UD ( $P < 0.05$ ). The V5 and V10 and V62 (*Pichia kudriavzevii*) promoted a higher rate of dry matter (DM) reduction of UD than other yeast and strain V5 was the most efficient in dry matter reduction of SB ( $P < 0.05$ ). The V5 and V62 were selected for analyzes of enzyme production, that showed V5 reducing pH at 96 h of fermentation and V62 and V5 increasing pectinase activate at 96 hs of fermentation. However, these isolates did not exhibit expressive xylanase activity under the evaluated fermentation conditions.

## Conclusion

The strain V5 promoted the highest dry matter reduction of sugarcane bagasse and *U. decumbens*, showing significative yeast biomass production. This yeast and the V62 strain increased the pectinase activities at 96 h of fermentation of *U. decumbens* showing potential of industrial application and for animal diets. Additionally, V5 strain would be selected and represent potential probiotic for cattle raised in tropical pasture

# Background

The complex ruminal microbioma may supply proteins, energy, and vitamins to the ruminants, which contribute to their growth and production. This ecosystem comprises known species of bacteria, filamentous fungi, and protozoa [1, 2]. Nevertheless, little is known about the yeast species in the rumen, that constitute up to 6.76 log of colony forming unit (CFU) per ml of ruminal fluid in healthy cattle of different ages [3–5].

Research evaluating the incorporation of *Saccharomyces cerevisiae* yeast from exogenous sources in the ruminant diet has shown a reduction in the overall ruminal oxygen content and increased ruminal bacterial populations and microbial protein production [6]. This yeast may reduce lactic acid levels, to

control the ruminal pH, and to favour cellulose digestion. This eukaryote also prevented diseases and reduced faecal excretion of pathogenic bacteria [7, 8]. However, some of the commercial strains of *S. cerevisiae* have exhibited limited growth in the rumen [9, 10].

The natural occurrence of yeast in the ruminal fluid has been reported for cattle fed with different sources of tropical roughage [3–4, 11–13]. Surprisingly, we detected a significantly higher population of yeast in adult cows raised on pastures of *Uroclhoa* spp. during the dry season than young cattle [4]. The characterisation of yeasts from the rumen of cattle that grazed on lignified pastures and having low nutritional value would support the selection of isolates that may facilitate the degradation of cell wall in roughages with high lignocellulose concentrations during dry seasons. In this study, our aims were to identify and evaluate the growth, biomass production, and enzymatic activity of yeast strains isolated from bovine rumen in submerged fermentations containing roughages with high concentrations of lignocellulose.

## Results

### Identification and molecular characterization of yeasts from bovine rumen

In this study were identified three yeast species from the rumen of the cow fed on lignified UD pasture, considering morphology and physiological tests and molecular analyses. Among the identified species, *Pichia kudriavzevii* was the most common, followed by *Rhodotorula mucilaginosa* and *Candida tropicalis* (Table 1). The phylogenetic analyzes of D1- D2 domain sequences of the 26S rRNA showed these isolates clustered with reference strains of the their respective species and intraspecific variations were not detected (Figure. 1).

### Degradation of lignocellulosic substrates

In fermentations containing SB, no significant differences were detected for cell mass production between the evaluated yeast strains (Table 2). However, for fermentations with UD, the strains of *R. mucilaginosa* (V16 and V10) had lower mass production than other strains ( $p < 0.05$ ). Yeast mass productions were significantly higher in fermentations using UD than SB ( $p < 0.001$ , Table 2).

The V5 and V10 and V62 (*Pichia kudriavzevii*) showed a higher rate of dry matter reduction of UD than other strains ( $p < 0.05$ ); however, the strain V5 was the most efficient in dry matter reduction of SB (Table 2). As expected, we detected a higher dry matter reduction of UD than SB by the yeast strains ( $p < 0.05$ ).

The strain V5 (*R. mucilaginosa*) showed higher biomass production in the medium containing UD and together with V62 promoted more reduction of final pH of fermentation medium in comparison to other strains ( $p < 0.05$ , Table 2). The strain V12 (*C. tropicalis*) increased the final pH of medium containing UD, possibly through its proteolytic action. We detected a low degradation rate of the evaluated substrates ( $< 3.99\%$ ).

In the second experiment, comparing the two selected strains, there were no significant variations of pH, xylanase and pectinase activities and in the total protein from the supernatants of the fermentations between these strains (Figures. 2, 3 and 4). The isolates did not exhibit expressive xylanase activity under the evaluated fermentation conditions. However, the regression analysis showed that V5 reduced pH at 96 h of fermentation (Figure. 3) and V62 and V5 increased the pectinase activities at 96 hs of fermentation (Figure. 4).

Table 1 Molecular identification by sequence analyses of D1/D2 variable domains of the large rDNA subunit of yeast from rumen of Zebu cattle raised in tropical pastures

Yeast Strains	Accession number *	Proposed identification	N° of bp analyzed	Identity (%)	Query coverage (%)	BLAST results [n° acc. GenBank] and n° strain from culture collection
V5	[MN380264]	Rhodotorula mucilaginosa	582	100	100	R. mucilaginosa DAMB1[MK968443.1]
10S	[MN380262]	Rhodotorula mucilaginosa	587	99	100	R. mucilaginosa SM03UFAM [MN268779.1]
16S	[MN380264]	Rhodotorula mucilaginosa	584	99	100	R. mucilaginosaenv1 [MN075224.1]
21S	[MN3802265]	Pichia kudriavzevii	580	100	100	P. kudriavzevii Z2 [MK310151.1]
25S	[MN380266]	Pichia kudriavzevii	579	100	100	P. kudriavzevii OE9 [LC487598.1]
V61	[MN380268]	Pichia kudriavzevii	579	100	100	P. kudriavzevii KKP 3005 [MK881743.1]
V62	[MN380269]	Pichia kudriavzevii	583	100	100	P. kudriavzevii CR-Y112 [KY273299.1]
12S	[MN380263]	Candida tropicalis	587	100	100	C. tropicalis A1 [MK409681.1]

\*Culture Collections registered in World Federation for Culture Collections. <sup>a</sup>D1/D2 sequences of yeast from Cow deposited.

Table 2

Mean of final pH, yeast biomass (mg), dry matter (dm) reduction and degradation rate (%) of sugarcane bagasse (SB) and *Urochloa decumbens* (UD) in submerged fermentation containing yeast from rumen fluid of Nelore cows

Strains	pH		yeast biomass (mg)		Dry matter reduction*		Degradation rate (%)**	
	SB	UD	SB	UD	SB	UD	SB	UD
<i>R. mucilaginosa</i>								
V5	6.2087 b	5.8250 c	16.1 a	27.3 a	0.1421 a	0.1733 a	3.16	3.99
V16S	6.5862 b	6.6950 a	14.1 a	13.4 b	0.1093 b	0.1604 b	0.31	0.40
V10S	6.5050 b	6.6950 a	10.5 a	14.0 b	0.1140 b	0.1741 a	0.58	1.45
<i>P.kudriavzevii</i>								
V21S	6.5875 b	6.6250 a	16.8 a	20.6 a	0.0976 b	0.1709 b	0.00	0.80
V62	6.0662 b	6.3000 b	17.5 a	23.0 a	0.0992 b	0.1728 a	0.00	1.43
V25S	6.5325 b	6.6750 a	16.7 a	23.4 a	0.0859 b	0.1677 b	0.31	1.05
V61	6.2250 b	6.5750 a	15.0 a	22.2 a	0.0789 b	0.1653 b	0.00	0.80
<i>C. tropicalis</i>								
V12S	7.1912 a	7.1600 a	19.3 a	26.4 a	0.0921 b	0.1612 b	0.00	0.40
Control	6.5758 b	6.7800 a	00.0 b	00.0 c	0.1140 b	0.1645 b	-	-
cv(%)	6.45	5.26	25.82	15.51	21.05	22.5	21.05	22.5
Note: Average with different letters in column are different by Scott-Kont test considering p 0.05; Coefficient of variation (cv) (%).								
Reduction of dm = 1- (final dm / initial dm)								
**Degradation rate = (dm reduction with yeast – dm reduction of control) x 100								

## Discussion

These three yeast species identified in this study have also been reported in the ruminal microbiota analysis of cattle fed with tropical forages [3–5]. *Pichia kudriavzevii* was also the most frequent yeast in the rumen of cattle from South of Minas Gerais, Brazil, which may be due to its ability to better adapt to ruminal conditions. All strains of this species showed growth in anaerobic conditions and in temperatures that predominate in the rumen [5].

The yeast *R. mucilaginosa* has also been detected in ruminal fluid samples from three fistulated cows and in the hay that was used to feed these animals [13]. In other study involving three fistulated Holstein cows, the researchers isolated yeast colonies and identified the Levica 18 strain, which was 98% similar to *R. mucilaginosa* [14]. The genus *Rhodotorula* is common in the environment and can be frequent in soil, water, milk, fruit juices and air samples [15]. This species has the capacity to assimilate glucose, sucrose and galactose [16].

Strains of *Candida tropicalis* were also identified in a study of fistulated cows fed with *Trifolium pratense* L. [13]. In a analyse conducted in northern Minas Gerais, Brazil, evaluating the rumen microbiota of goats fed with tropical pasture, 90% of the total yeast isolated corresponded to the species *Pichia membranifaciens* and 10% to *C. tropicalis* [17]. A strain of *C. tropicalis* (BPU1) was also isolated from the rumen of the Malabari goat, showing dual production of biosurfactant and polyhydroxybutyrate in a simple mineral salt medium, using vegetable oil as the sole carbon source [18].

The occurrence of yeast in the rumen environment is still poorly supported in the scientific literature despite their high population, especially in adult cows raised on low quality pastures [4]. Studies associated with the rumen microbiota have frequently ignored the presence of these yeast fungi in the rumen ecosystem [5]. Although the three yeast species identified in this study have also been reported in other studies of this ruminant site, the role of these microorganisms should be better elucidated in the ruminal ecosystem of animals of different categories fed with different diets.

In a review, we verified that these yeast species are potential producers of enzymes involved in the oxidative degradation of lignocellulosic biomass such as superoxide dismutase and peroxidase based on its information available from the UniProt p database (Table 3) [19–21]. Genes coding for enzymes that act on the degradation of hemicellulose and lignin as 1,3- $\beta$ -glycosidases, mannosidases, trehalases, esterases and deacetylases were reported in *P. kudriavzevii*, [19, 20]. Sequencing of the complete genome of this species revealed that yeast has the potential for fermentation of xylose, xylitol dehydrogenase and xylulokinase, enzymes that are considered important in the production of second-generation ethanol, as reported by Chan et al., [19].

The cultivation conditions employed in this study were not favourable to a substantial production of the fibrolite enzymes, possibly due to the low nutrient contents of the lignocellulolytic substrates and of the minimum culture medium. Hence, future studies should focus on adding specific nutrients and promoting other cultivation conditions that would favour the better expression of enzymes and metabolites that would improve the degradation of these lignocellulose-containing materials. In this study, the strain V5 (*R. Mucilaginosa*, a non-pathogenic species) might be an ideal strain to be selected and evaluated with

further tests to improve the digestibility of UD and to represent a natural yeast probiotic for cattle raised in tropical pasture.

Table 3

Description of the genes coding for carbohydrate-active enzymes noted in the UniProt database for the *Rhodotorula mucilaginosa* and *Pichia kudriavzevii*

Yeast species	Enzymes	Genes
R. mucilaginosa	Superoxide dismutase (EC 1.15.1.1)	SOD
	Glutathione peroxidase	-
	Epoxide hydrolase	EPH1
	Putative epoxide hydrolase (Fragment)	EPH1
P. kudriavzevii	Superoxide dismutase [Cu-Zn] (EC 1.15.1.1)	BOH78_3317JL09_g3534
	Superoxide dismutase [Fe] 2. chloroplastic	BOH78_4383
	Putative glycosidase crf2	BOH78_3033
	Glucosidase 2 subunit alpha	BOH78_2458
	Glucan 1.3-beta-glucosidase	BOH78_2642
	Mannanendo-1.6-alpha-mannosidase(EC 3.2.1.101)	BOH78_3729
	Glucosidase YgjK	BOH78_2824
	Superoxide dismutase (EC 1.15.1.1)	BOH78_1681
	Trehalase (EC 3.2.1.28) (Alpha-trehalose glucohydrolase)	BOH78_5026
	Cell surface superoxide dismutase [Cu-Zn] 6	BOH78_1067
	Glucan 1.3-beta-glucosidase	BOH78_1483
	Putative N-acetylglucosamine-6-phosphate deacetylase	BOH78_5404
	Putative beta-glucosidase btgE	BOH78_3217
	Alpha-mannosidase	BOH78_5454
	alpha-1.2-Mannosidase (EC 3.2.1.-)	BOH78_1133
	alpha-1.2-Mannosidase (EC 3.2.1.-)	BOH78_2213
	Glucan 1.3-beta-glucosidase	BOH78_3239
	General alpha-glucoside permease	BOH78_4431
	Mannosyl-oligosaccharide glucosidase	BOH78_2180
	Putative glycosidase CRH1	BOH78_1259



Yeast species	Enzymes	Genes
	Alpha-mannosidase (EC 3.2.1.-)	BOH78_4475
	Pseudouridine-5'-phosphate glycosidase	BOH78_2691
	Putative N-acetylglucosamine-6-phosphate deacetylase Glucosidase 2 subunit beta	BOH78_2301 BOH78_1018
	Putative secreted beta-glucosidase SUN4	BOH78_3462
	Trehalase (EC 3.2.1.28) (Alpha-trehalose glucohydrolase)	JL09_g2484
	alpha-1.2-Mannosidase (EC 3.2.1.-)	JL09_g645
	Putative secreted beta-glucosidase adg3	BOH78_3821
	Mannan endo-1.6-alpha-mannosidase (EC 3.2.1.101)	JL09_g2280
	Alpha-mannosidase (EC 3.2.1.-)	JL09_g3731
	alpha-1.2-Mannosidase (EC 3.2.1.-)	JL09_g1714
	Putative N-acetylglucosaminyl-phosphatidylinositol de-N-acetylase	BOH78_0024
	Mannan endo-1.6-alpha-mannosidase (EC 3.2.1.101)	BOH78_3728 JL09_g2281
	Glucan 1.3-beta-glucosidase	BOH78_2598 JL09_g2390
	Mannan endo-1.6-alpha-mannosidase (EC 3.2.1.101)	BOH78_4436 JL09_g171
	6-phosphogluconolactonase 3	BOH78_0239 JL09_g819
	D-arabinono-1.4-lactone oxidase	BOH78_3945 JL09_g3239

## Conclusions

The strain V5 promoted the highest dry matter reduction of sugarcane bagasse and *U. decumbens*, showing expressive yeast biomass production. The inclusion of V5 or V62 strains increased the pectinase activities at 96 h of fermentation of *U. decumbens*, showing potential of biotechnological application and for animal diets.

## Methods

Isolates of yeast from rumen

Eight yeast strains isolated from the rumen of Nelore cows reared in *U. decumbens* lignified pasture in northern Minas Gerais were evaluated for degradation of two roughages. These microorganisms were collected by puncture of the ventral rumen sac and isolated in our previous study [4] and were selected because of their high populations in the rumen environment of adult beef cows (> 6 log CFU per ml of ruminal fluid). These strains were grown in Sabouraud broth and stored in an ultra-freezer at -80° C and deposited and available in the Yeast Culture Collection of the Institute of Agricultural Sciences of the Universidade Federal de Minas Gerais.

### Characterization and identification

Preliminarily we grouped the yeast strains according to colony morphology and physiological tests performed according to the procedures described by Kurtzman et al. [22]. For molecular analyses, the yeast isolates of rumen were grown on Sabouraud agar for seven days, and DNA was extracted according to a method previously described by Rosa et al., [23]. The D1/D2 region of rDNA was amplified by polymerase chain reaction (PCR) using primers NL1 (5'-GCA TAT CAA AAG GAA GAG TAA GCC-3 ') and NL4 (5'- GGT AAG CTT CGC TGT CCG G-3'), according to a method previously described by White et al., [24]. The amplified product was quantified with a NanoDrop 1000ND (NanoDrop Technologies) and the concentration was adjusted to 100 ng  $\mu\text{L}^{-1}$  for use in sequencing reactions.

Sequencing was performed with DYEnamic (Amersham Biosciences, USA) in a Mega-BACE 1000 automated sequencing system at the Genome Analysis Center and Gene Expression. The rDNA sequences were analysed using BLASTn (v.2.215) of BLAST 2.0 at the National Center for Biotechnology Information (NCBI) website [25]. Conspecific strains differed by no more than three among the 500–600 nucleotides of the D1/D2 domains and the isolates with 99% sequence similarity to deposited sequences were considered as the same species [22].

### Phylogenetic analyzes

The D1- D2 variable domain sequences of the 26S rRNA gene from the yeast isolates were used to reconstruct their phylogenies using the MEGA X version 10.1 (BETA) program [26]. Analyzes were performed individually with the sequences belonging to the Basidiomycota and Ascomycota, which were aligned using the Clustal W method [27]. For each genus, the alignments were performed by including sequences of other yeast strains deposited in GenBank. Akaike's information criterion was used to identify the most appropriate evolution model for the dataset of each phylum.

These datasets were estimated by the Maximum Likelihood Method, based on the Tamura-Nei model [28] and discrete Gamma distribution was used to model the evolutionary rate differences between sites [5 categories (+ G, parameter = 0.4430)] for Basidiomycota and [5 categories (+ G, parameter = 0.3043)] for Ascomycota. Tree robustness was estimated by bootstrap analysis with 1000 replicates [29] and all nucleotide sequences were submitted to GenBank, assigned MN380262 to MN380269 accession numbers.

## Lignocellulosic materials

The material of *Urochloa decumbens* (UD) hay was collected during the dry season (March to June) on a farm located in the Montes Claros region, Northern Minas Gerais, Brazil and the sugarcane bagasse (SB) was provided by alcohol and sugar company São Judas (SADABio), located in the Jaíba city at same region. The species of these forages were identified according to their morphological characteristics expressed in the flowering periods [30].

These fibrous materials were dried and grounded in a Willey knife mill to 1–3 mm particle size. Subsequently, subsamples were subjected to chemical composition analysis [30] as described in Table 4.

Table 4  
Chemical composition of *Urochloa decumbens* hay of sugarcane bagasse utilized in this study

Ingredients	<i>Urochloa decumbens</i>	Sugarcane bagasse
Dry matter (DM)	95.38	97.16
Neutral detergent fiber (NDF) (% in DM)	82.26	86.89
Acid detergent fiber (% in DM)	53.04	57.89
Lignin (% in DM)	7.50	6.15
Crude protein (% in DM)	3.06	2.25
Ether extract (% in DM)	1.02	0.08
Minerals (% in DM)	5.89	3.36

## Fermentations

In a first experiment, submerged fermentation occurred in tubes with 30 mL of liquid medium (medium C) containing 5 grams of ammonium sulphate, 1 gram of potassium monobasic phosphate, 0.5 grams of heptahydrate magnesium sulphate and 0.33 grams of carbon source (SB or UD as sole carbon sources) per litre. The roughage samples were weighed on an analytical balance and packed in non-woven textile (NWT made of polypropylene polymer) bags (3 × 3 cm) and added to the culture medium for further autoclaving.

The inoculums of each yeast strain were prepared after incubation in Sabouraud agar medium (Acumedia, Lansing, Michigan) plus chloramphenicol (150 mg / l) for 48 hours. Subsequently, yeast colony masses were added and incubated in Sabouraud broth at 37°C for 48 hours. After this period, 3 mL of inoculum containing approximately  $3.2 \times 10^4$  CFU / mL was added to tubes containing 30 mL of C medium that contained the lignocellulosic substrates SB or UD. In the control tubes, same volume of the culture medium with no inoculum was added.

The tubes were incubated in a shaker (NT 715, Novatécnica, São Paulo, Brazil) at 37 °C and 150 RPM for seven days. The pH of the medium was measured before and after incubation, using a digital potentiometer (pH 1800, Waterproof Pen pH Tester, Instrutherm, São Paulo, Brazil).

After this period, the tubes were centrifuged to separate the yeast mass, which, together with the non-woven textile (NWT – 100 g/m<sup>2</sup>) bags., were kept in a circulating oven (TE-394/3, Tecnal, São Paulo, Brazil) for four days at 40 °C until they presented constant weight. Subsequently, the dry yeast mass and dry matters of SB or UD were obtained using a moisture determinant (MOC63u Shimadzu, Kyoto, Japan).

### Production of enzyme extracts

In the second experiment, the strains V5 (*Rhodotorula mucilaginosa*) and V62 (*Pichia kudriavzevii*) were selected, considering their higher degradation rates of UD and reduction of pH by them in the first experiment. Submerged fermentation occurred as previously described, comparing the two sectioned yeast strains and the two carbon sources (SB or UD) at 0, 48, 96, 144 and 192 hours of incubation.

After each fermentation period, the tubes containing the yeast and their controls were centrifuged at 11,000 g for 30 minutes at 4 °C (Centrifuge 5804 R, Eppendorf AG Brazil) for the detection of enzymes and the total protein of the supernatants. The extracts were placed in 40 mL flasks and stored in a vertical ultra-freezer at -80 °C (AmericanLab, São Paulo - Brazil) until the time of analysis.

### Enzymatic activities

To evaluate xylanase activity of the extracts, mixtures containing 1 mL phosphate buffer (0.1 M; pH 6.8), 0.5 mL supernatant and 0.5 mL xylan (D-xylana, Vetec, Brazil, Sao Paulo) at 0.25% were prepared. The polygalacturonase (pectinase) activity was evaluated after mixing 1 mL phosphate buffer (0.1 M; pH 6.8); 1 mL of supernatant and 2 mL of polygalacturonic acid (Pectin - Sigma Aldrich, Brazil, Sao Paulo) (0.3%) dissolved in 5.0 mM sodium acetate buffer pH 4.5 and 0.1 M NaCl. The reactions were performed, respectively, for 15 and 20 min at 39.0 °C.

After the reaction, total amount of reducing sugars were determined by adding 1 mL of the mixtures to 1 mL of dinitrosalicylic acid. The tubes were shaken and heated in a water bath (SP06 / 100ED, SPLabor, Sao Paulo, Brazil) at 96 °C for five minutes. Subsequently, the tubes were cooled in an ice bath for five minutes, and then 16 mL of double potassium sodium tartrate was added. The blanks from each reading were performed with the enzymatic extracts evaluated without the addition of the respective carbohydrates to be degraded.

Absorbance was measured on a spectrophotometer (AJX1000, Ajmicronal, São Paulo, Brazil) at 540 nm, using distilled water as a control. Data were recorded using the M.Wave Basic (ChromTech) program and glucose concentrations were expressed in g/L. One unit of enzymatic activity was defined as the amount of enzyme required to release one  $\mu\text{mol}$  of product per minute under the evaluated experimental conditions.

Total protein concentration was estimated using the Bradford method, where 1 mL of Bradford reagent (Amresco®, Ohio, USA) and 100 µL of enzyme extract were added to test tubes, the reaction was processed for two minutes. A standard serum albumin curve was constructed at 25 °C using the M.Wave Basic (ChromTech) program. After the reaction, readings were taken in the spectrophotometer at a wavelength of 595 nm and quantifications were expressed in µg/mL, after reduction of the values observed in the respective fermentations without the yeast strains.

### Statistical analysis

This first experiment was conducted in a 2 × 9 factorial design with four replications, comparing the eight yeast isolates and the control (without microorganisms), and the degradation of the two substrates (UD or SB). The variables observed were pH, dry yeast mass, dry matter (dm) of lignocellulosic material residues and their degradation rate.

Reduction of dm = 1 - (final dm / initial dm)

Degradation rate = (dm reduction with yeast – dm reduction of control) x 100

In the second experiment, a 2 × 3 × 4 factorial design was performed to compare the two carbon sources, the two yeast strains selected from the first experiment and the control without yeast, in four periods, with four replications. The variables analysed were pH, pectinase and xylanase activity and total protein production. After normality and homogeneity test, the data were subjected to analysis of variance test and the means were compared to a 5% of significance. Data were analysed also by regression analyses using the SAEG statistical package, Version 9.1

## Abbreviation

**CFU:** colony forming unit, **SD:** sugarcane bagasse, **UD:** *Urochloa decumbens*, **DM:** dry matter, **NDF:** Neutral detergent fiber.

## Declarations

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### **Availability of data and materials**

The datasets used and/or analyzed during the current study and yeast strains are available from the corresponding author on reasonable request

### **Authors' contributions**

ERD conceived, designed and coordinated the study. SML, JCN, HARM, CES, ADLJ participated in data collection, analyses and drafting the manuscript. ERD, JCS and VLS analyzed, wrote and submitted the manuscript for publication. All the authors revised and approved the final manuscript.

### **Ethical Approval**

The yeast isolates were collected in previous experiments submitted and approved by the Ethics Committee on Animal Experiments of the UFMG (protocol n<sup>o</sup> 156/05), regulated by the National Council for Control of Animal Experimentation of Brazil regulated by the National Council for Control of Animal Experimentation of Brazil. The authorization for the study of the yeast and plant species collected was approved by National Management System of Brazil Genetic Heritage and Associated Traditional Knowledge (cadastres AC14923, ABAA22D and A3B530C).

### **Consent for publication**

Not applicable.

### **Competing interests.**

The authors of this manuscript have no financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

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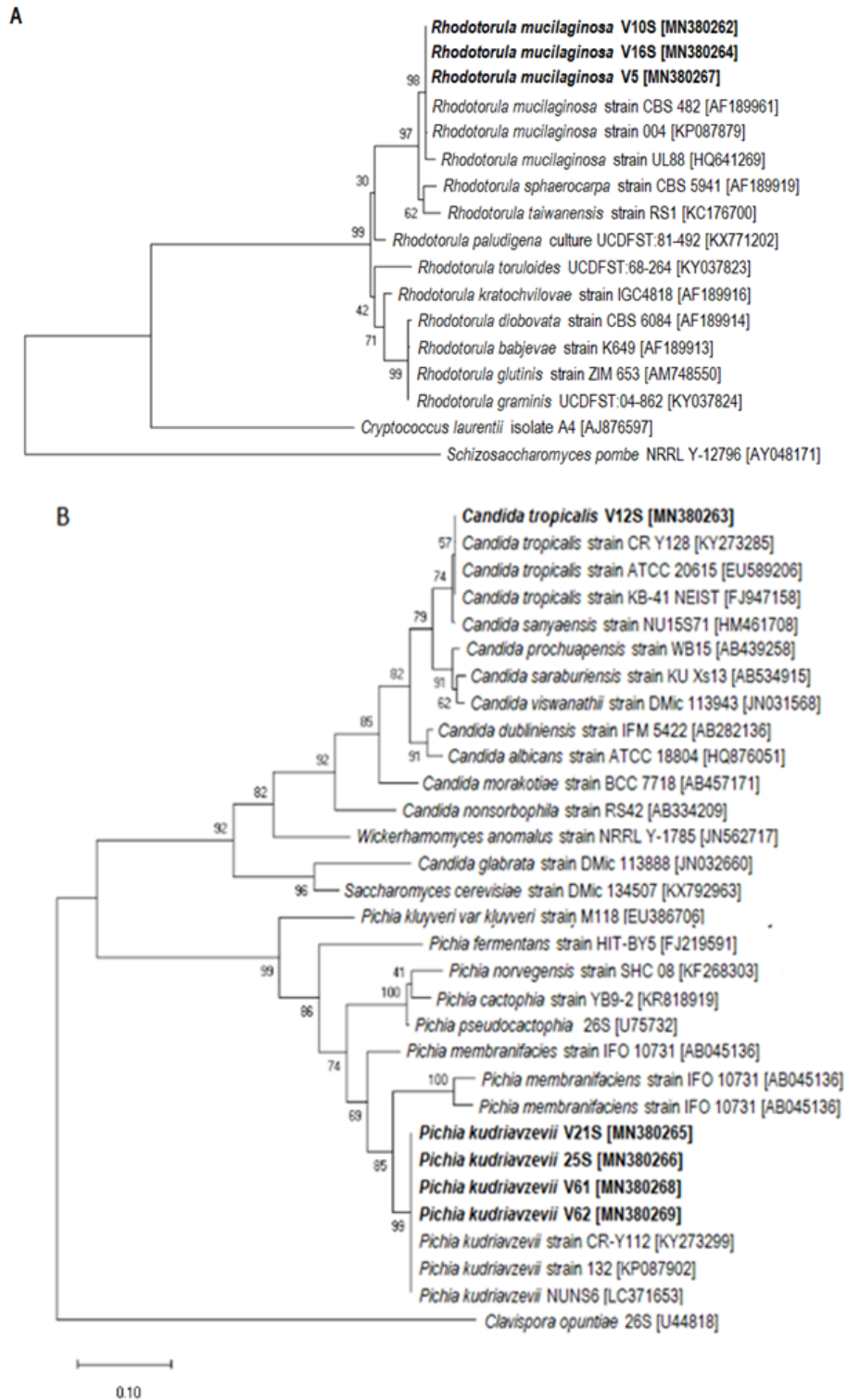
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## Figures

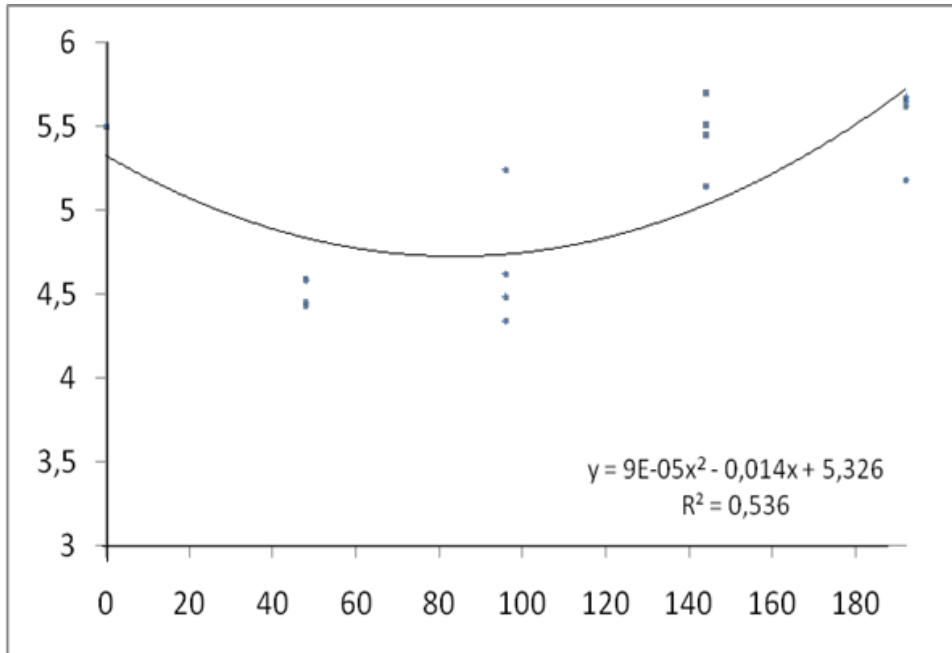




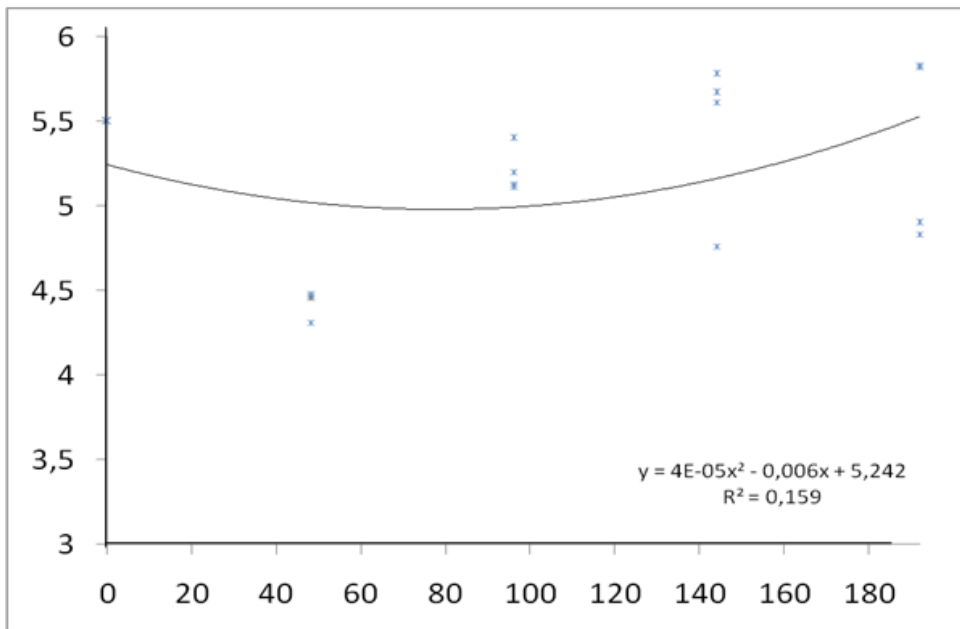
**Figure 1**

Consensus trees representing phylogenetic analyzes of the sequences of the D1- D2 variable domain (26S rRNA gene) of yeast isolated from the rumen of Zebu cows from phylum A) Basidiomycota and B) Ascomycota, comparing to sequences of their species and strains more closet and relevant deposited in GenBank. The isolated names and access codes are in bold. The tree containing the Basidiomycota yeast sequences was rooted using the *Schizosaccharomyces pombe* NRRL Y-12796 [AY048171] isolate as an

external group and the tree containing the Ascomycota yeast sequences using the *Clavispora opuntiae* 26S isolate [U44818].



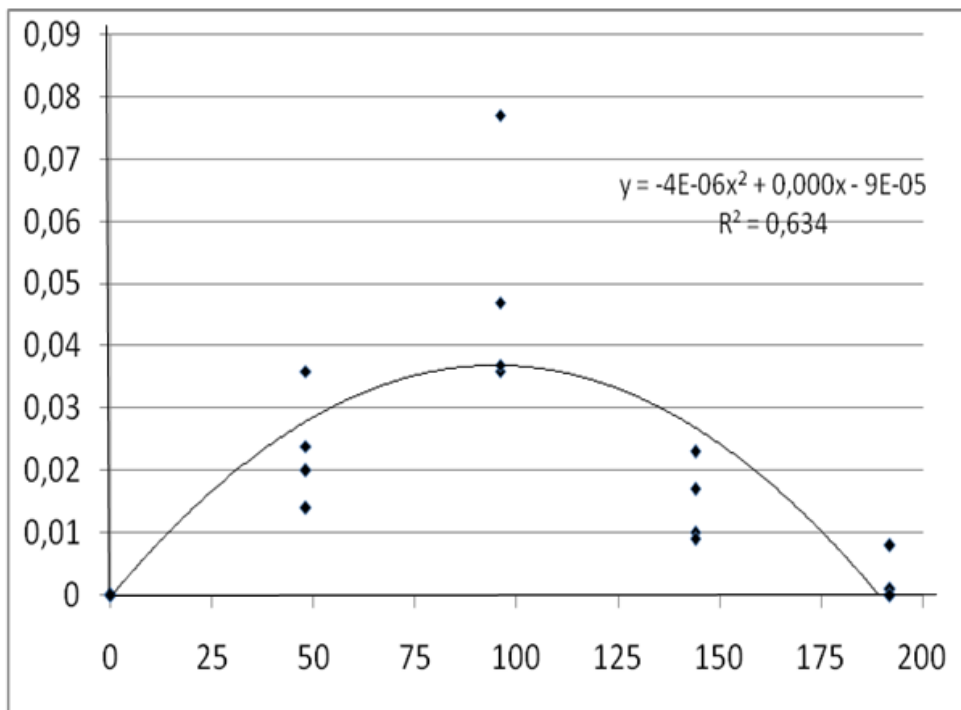
a) Periods (hours)



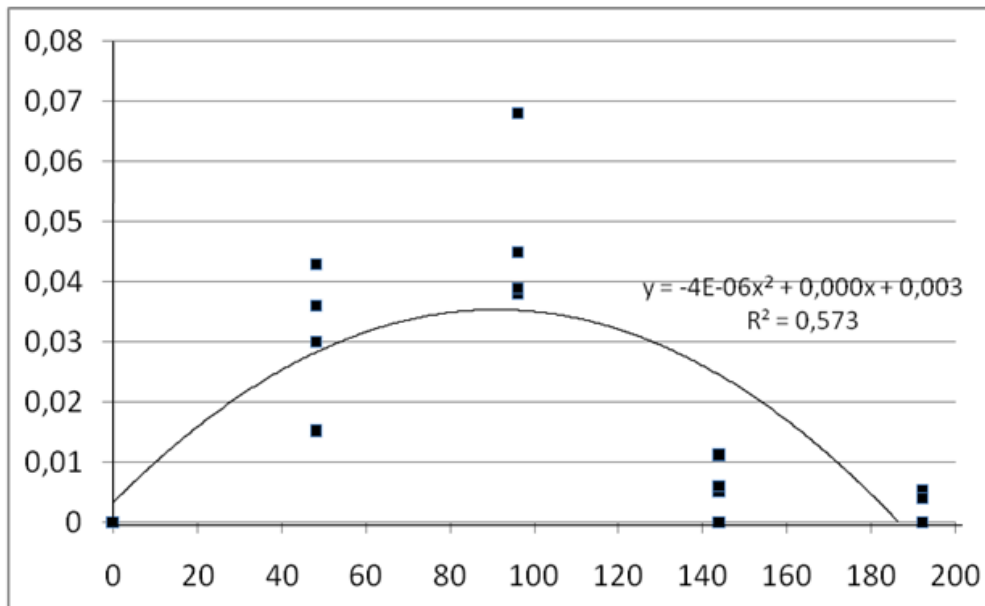
b) Periods (hours)

Figure 2

pH variation in submerged fermentations containing *Urochloa decubens* inoculated with *Rhodotorula mucilaginosa* (strain V62 (a)) and *Pichia kudriavzevii* (strain V5) (b)



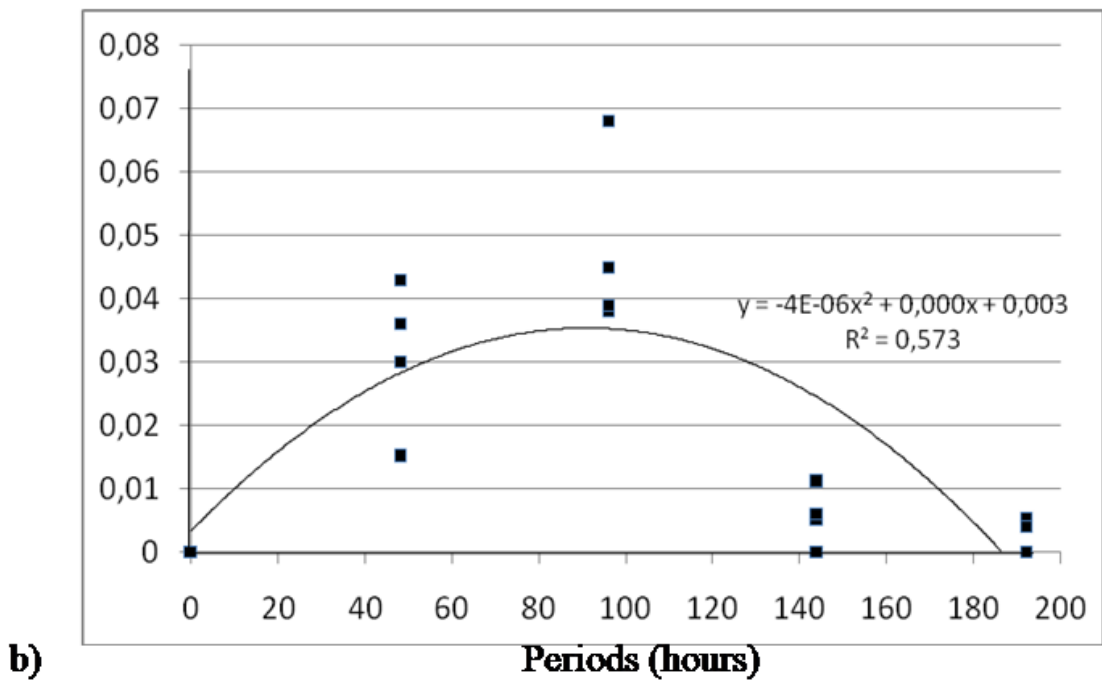
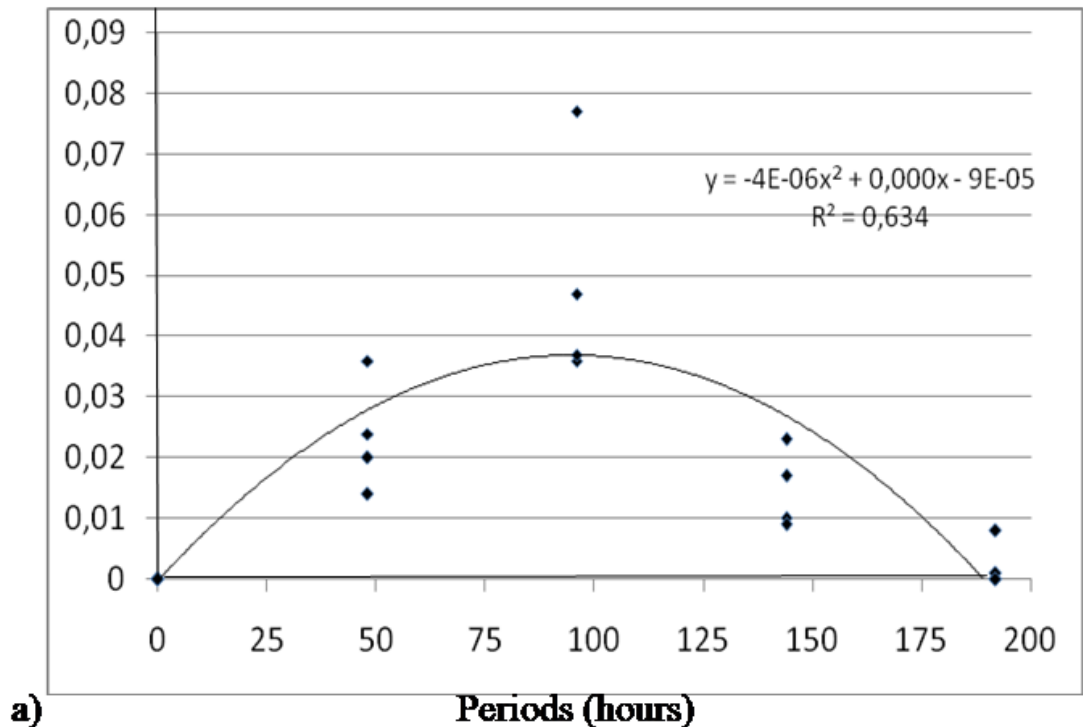
**a)** Periods (hours)



**b)** Periods (hours)

**Figure 3**

Protein production (g/L) in submerged fermentations containing *Urochloa decubens* inoculated with *Rhodotorula mucilaginosa* (strain V62 (a)) and *Pichia kudriavzevii* (strain V5) (b))



**Figure 4**

Pectinase production (g/L) in submerged fermentations containing *Urochloa decubens* inoculated with *Rhodotorula mucilaginosa* (strain V62 (a)) and *Pichia kudriavzevii* (strain V5) (b)