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Bio-saccharification and fermentation process of a non-conventional starchy material with isolates of autochthonous strains

Bio-sacarificación y fermentación de una materia prima no convencional rica en almidón utilizando microorganismos autóctonos

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Abstract
Consolidated bio-saccharification (CBS) is a promising technique for converting complex materials like starchy biomass into ethanol through simultaneous liquefaction and saccharification, leading to a more sustainable process. This study developed sequential bio-saccharification and fermentation of *Brosimum alicastrum* seed flour (BSF) in a single reactor using native microorganisms from *B. alicastrum* fruits. The native yeasts were identified through molecular techniques and their amylolytic capacity, growth rate at different temperatures, and ethanol tolerance were assessed, along with the basidiomycete *Trametes hirsuta* RT-1. The identified yeasts were *Candida tropicalis* (PL-1), *Pichia kudriavzevii* (TL-2), *Hanseniaspora guilliermondii* (TL-3), and *Meyerozyma caribbica* (RSL-4). *C. tropicalis* (PL-1) showed cell growth up to 42°C, 8% v/v ethanol tolerance, and partial BSF degradation, while *T. hirsuta* RT-1 showed 3% v/v ethanol tolerance and produced the enzymes α-amylase (49.77 ± 7.54 U/mL) and laccase (8,920 ± 1,236 U/mL). The highest ethanol production (18.93 ± 1.78 g/L) was achieved after 11 days of CBS using both *C. tropicalis* (PL-1) and *T. hirsuta* RT-1. This strategy represents a novel approach for bioethanol production using CBS from an available non-conventional and inexpensive material, without the use of commercial enzymes or microorganisms.
Keywords: *Trametes hirsuta*; *Candida tropicalis*, Amylase, *Brosimum alicastrum*.

Resumen
La bio-sacarificación consolidada (CBS) es una técnica prometedora para producir etanol de manera más sostenible a partir de materiales complejos con alto contenido de almidón que requieren licuefacción y sacarificación simultáneas. Este estudio desarrolló una sacarificación y fermentación simultánea de harina de semillas de *Brosimum alicastrum* (BSF) en un solo reactor utilizando microorganismos nativos de frutas de *B. alicastrum*. Estos microorganismos fueron identificados molecularmente y se evaluó su capacidad amilolítica, tasa de crecimiento a diferentes temperaturas y tolerancia al etanol. Las levaduras identificadas fueron *Candida tropicalis* (PL-1), *Pichia kudriavzevii* (TL-2), *Hanseniaspora guilliermondii* (TL-3) y *Meyerozyma caribbica* (RSL-4). *C. tropicalis* (PL-1) mostró crecimiento hasta 42°C, tolerancia al etanol del 8% v/v y degradación parcial de BSF, mientras que *T. hirsuta* RT-1 presentó tolerancia al etanol del 3% v/v y producción de α-amilasa (49.77 ± 7.54 U/mL) y lacasa (8,920 ± 1,236 U/mL). La mayor producción de etanol fue de 18.93 ± 1.78 g/L y se obtuvo tras 11 días de CBS utilizando *C. tropicalis* (PL-1) y *T. hirsuta* RT-1. Esta estrategia representa un enfoque innovador para la producción de bioetanol utilizando un material disponible en la región, económico y no convencional mediante CBS sin necesidad de enzimas o microorganismos comerciales.
Palabras clave: *Trametes hirsuta*; *Candida tropicalis*, Amilasa, *Brosimum alicastrum*.

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1 Introduction

Bioethanol can be produced from competing food crops, lignocellulosic materials, or algal biomass. Bioethanol is classified as first, second, or third generation depending on the substrate from which it is produced (Ferreira *et al*., 2019). The main disadvantage of first-generation bioethanol is the ethical dilemma "food versus fuel". However, the technological infrastructure required for its synthesis is well understood and has proven to be a significant asset. This technological knowledge reduces ambiguity surrounding essential raw materials, the processing thereof, and market dynamics (Lennartsson *et al*., 2014). The raw materials employed in the production of first-generation bioethanol include crops which contain starch, with corn constituting the predominant source. Starch, the second most abundant biopolymer in higher plants after cellulose, plays a primary role in energy storage.

Starchy materials are mainly destined for fresh consumption, and a low percentage of the total available is processed into flours and starches (Suárez-Castillo *et al*., 2024). The use of non-edible starchy sources could be an interesting alternative for bioethanol production since it is a cheap and non-toxic renewable carbon source (Bušić *et al*., 2018; Satyanarayana, 2009). Despite the advantages of the use of starch biomass, process technologies must be applied to improve its functional properties in order to make it suitable for extensive use in various industries (Ramos-Villacob *et al*., 2024).

For bioethanol production from starchy biomass, two primary stages are involved: liquefaction and saccharification, in which, the use of amylases, such as α-amylase and glucoamylase, are essential to breakdown the starch into fermentable sugars. Subsequent to these stages, a fermentation step is initiated, wherein a yeast strain, usually Saccharomyces cerevisiae, is added to convert these sugars into ethanol (Zabed *et al*., 2017).

The use of commercial amylases in the starch-to-ethanol process has been identified as a significant factor in increasing the costs of saccharification, making this step one of the most expensive in the entire process (Favaro *et al*., 2019). Achieving economic viability is imperative for the sustainable production of biofuels, in considering of which several bioconversion strategies have been studied with a view to improving the cost-effectiveness of the saccharification. Consolidated biosaccharification (CBS) and consolidated bioprocess (CBP) are promising technologies for the effective conversion of biomass into bioethanol. CBP involves three simultaneous processes in a single reactor, which are enzyme production, saccharification, and fermentation. As previously mentioned, enzyme production is one of the most expensive steps in bioethanol production. Therefore, CBP could help to make this process profitable compared to conventional technologies by reducing the cost of enzyme procurement. Other advantage of this approach is the use of a single reactor, which can result in lower maintenance and operating costs. However, the main disadvantage is that the processes have different operating conditions, making optimization complicated.

In contrast, consolidating biosaccharification (CBS) involves the use of microorganisms that can produce enzyme cocktails, which catalyze the conversion of biomass and the hydrolysis of simple carbohydrates (Jahangeer *et al*., 2024). This technology entails in the production of enzymes and their subsequent application for saccharification within the same reactor, but the use of native strains can enhance the bioconversion of starch into fermentable sugars and facilitate the fermentation process. This approach has the potential to reduce the cost of bioethanol.

Yeasts currently employed for fermentation have not been carefully selected for the feedstock, but rather, have been used due their historical background (Steensels *et al*., 2014; Umeh *et al*., 2017). The use of native microorganisms has the potential to enhance the cost-effectiveness of bioethanol production, given that native microorganisms are inherently adapted to the local conditions in which they have evolved although they are rarely used in current industrial processes. Several autochthonous yeasts isolated from dates, fruits, sugarcane, and beet molasses crops have demonstrated the potential to enhance bioethanol yields compared to commercial strains (Kechkar et al., 2019). Our research group has identified and studied a native basidiomycete strain, *Trametes hirsuta* Bm-2, which has amylolytic and fermentative capabilities that allow its use in a consolidated bioprocess using a starchy substrate (Olguin-Maciel *et al*., 2019). The pursuit of hyper-productive strains capable of improving hydrolysis and fermentation processes has gained significant prominence, highlighting the necessity to identify novel microorganisms for bioconversion processes.

In this study, the use of *Brosimum alicastrum* Sw. (ramon tree) seed flour is proposed for the production of bioethanol, since it is not currently regarded as a food crop. This tree is native to the Mesoamerican and Caribbean regions; it grows naturally in several Mexican states, including the Yucatan Peninsula; and it also exhibits a remarkable ability to grow in soils with limited nutrients, a trait that contributes to its resilience in different climates. Ramon seeds contain proteins, lipids, calcium, vitamins (A, B, C), fiber, and most importantly a high carbohydrate content (75%),

with approximately 63% of this carbohydrate being starch, which can be transformed into bioethanol. In addition, phenolic compounds, such as gallic, vanillic, caffeic, *p*-coumaric, hydroxybenzoic and chlorogenic acids, have also been identified. These compounds can act as natural redox mediators for enzymes or as substrates for laccase production, thereby facilitating a sustainable exploitation of the material within a biorefinery approach (Meiners *et al*., 2009; Olguin-Maciel *et al*., 2017). The annual seed production per tree is estimated to be 95 kilograms, and this estimate indicates an annual production of 28.6 tons in a plantation with 300 trees per hectare, or 19.7 tons in a plantation with 200 trees per hectare (Hernández-González *et al*., 2014).

The tree also provides important environmental benefits, including the protection of soil, bodies of water, and biodiversity, thus making it a promising species for restoration. Additionally, its slow growth rate contributes to its role as a carbon sink, making it a suitable option for carbon capture programs to combat global warming (Meiners *et al*., 2009). Historical studies suggest that this species was cultivated by the Maya civilization and other Mesoamerican peoples as a staple food and for medicinal purposes. However, BSF is currently an underexploited resource and is rarely used for human consumption in the Yucatan Peninsula (Losoya-Sifuentes *et al*., 2023; Ozer 2017; Pérez-Pacheco et al., 2014). Consequently, the exploitation of the ramon tree is an opportunity to preserve and utilize it for the benefit of ecosystems and rural development, and for the sustainable production of biofuels and value-added compounds, such as phenolic compounds and valuable enzymes (laccase and amylase).

The aim of this study was to develop a sequential bio-saccharification and fermentation process in a single reactor for the bioconversion of *Brosimum* seed flour (BSF) using autochthonous microorganisms: a fungus for liquefaction and saccharification and isolated yeasts from the ramon fruit that were identified through a polyphasic approach for fermentation.

2 Materials and methods

2.1 Raw material

Seeds of *B. alicastrum* were collected from Caucel, Yucatan, Mexico (21°00'53"N 89°42'25"W). The husked seeds were dried in a convection oven (Binder, Fed model 115 ®, Tuttlingen, Germany) at 70°C for 72 h and stored in a desiccator until milling to obtain BSF as previously described by Pérez-Pacheco *et al*., (2014). The BSF used in this work contained 75% carbohydrates of which 63% was starch and 12.24 % were total proteins (Olguin-Maciel *et al*., 2020).

2.2 Identification of yeast cultures and fungal strain

The four native yeasts used in the present study were isolated from the pericarp (P), coat (T) or seed (S) of ramon fruits (Huchin Poot, 2015) and conserved in YPD (Yeast Extract Peptone Dextrose) media for further analysis. They were morphologically identified as *Candida tropicalis* (PL-1), *Pichia kudriavzevii* (TL-2), *Hanseniaspora guilliermondii* (TL-3) and *Meyerozyma caribbica* (RSL-4), using a taxonomy analysis following Kurtzman *et al*. (1990) and Barnett *et al* (2011).

Besides the taxonomical analysis a molecular identification was carried out, and the total genomic DNA was extracted using the methodology proposed by Tapia-Tussell *et al*. (2006). DNA concentration and purity were determined in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Seattle, WA, USA); then, D1/D2 Domain LSU 26S rDNA was amplified by PCR using the universal primers NL1 (5´-GCA TAT CAA TAA GCG GAG GAA-3´) and NL4 (5´-GGT CCG TGT TTC AAG ACG G-3´) (Libkind *et al*., 2003). Sequences were obtained by Macrogen Inc, Korea, processed with BioEdit Program v 7.0.5 (Hall, 1999), and analyzed using Molecular Evolutionary Genetic Analysis (MEGA) software version 5.0 (Kumar *et al*., 2018). A tree was built using the neighbor-joining method and the unweighted pair group method with arithmetic mean (UPGMA). The relative support of clustering was evaluated by bootstrap analysis (1000 resamples) using *Kluyveromyces maxianus* as the out-group.

Meanwhile, the native fungal strain isolate was obtained from the endemic plant *Accalypha gaumeri* in Tinum, Yucatan, Mexico (20°42´00´´N, 88°29´00´´W). It was identified at species level as *Trametes hirsuta*, by the amplification of the 5.8S-ITS regions using the universal primers ITS1 and ITS4 (White *et al*., 1990). The sequence of the fungus was registered at GenBank with accession number OM732416.1.

2.2.1 Yeast conservation

The yeasts were preserved in distilled water suspensions and in 25% glycerol-YPD broth at -80°C in the Laboratory of Micromycetes C006, Institute of Biology, National Autonomous University of Mexico (UNAM) and were registered in the Fungal Collection of the Herbario Nacional (MEXU-UNAM) with codes MEXU 30461 for *C. tropicalis* PL-1, MEXU 30662 for *P. kudriavzevii* TL-2, MEXU 30463 for *H. guilliermondii* (TL-3) and MEXU 30464 for *M. caribbica* (RSL-4).

2.3 Inoculum preparation and assays

An inoculum of each native isolate was prepared to evaluate their amylolytic capacity, thermotolerance and tolerance to different concentrations of ethanol. This evaluation was used to select the most suitable isolate for the hydrolysis, saccharification and fermentation process. The inoculums were prepared by transferring colonies from YPD cultures with 24 h of incubation, into 10 mL sterile distilled water. The inoculums were incubated at 32 ± 2°C until the standard McFarland turbidity pattern (bioMerieux, Lyon, France) reached a value of 3.

Meanwhile, plugs of 1 cm diameter from a 5-days old PDA (BD Difco Potato-dextrose agar, Becton-Dickinson & Co., Sparks, MD, USA) culture with *T. hirsuta* (RT-1) were added into a 5% (w/v) solution of RSF, and where then it was incubated at 32 ± 2°C for 7 days. α-amylase and laccase activity were determined from the fungal inoculum.

2.3.1 Amylolytic capacity

The amylolytic capacities of the native isolates were tested into 16 mm tubes containing 4.5 mL soluble starch solution (0.5% (w/v) Sigma-Aldrich) with the addition of 0.5 mL of BD Difco Nitrogen Base solution (6.7g DNB/100 mL distilled water) (Wickerham, 1951). The tubes were inoculated with 0.1 mL of the inoculum, and incubated for two weeks at 32 ± 2°C. Subsequently, two drops of Lugol solution were added to each tube to evaluate starch hydrolysis according to the color change of the reagent; from dark violet to: a) to pale yellow, meaning complete hydrolysis; b) to light violet or reddish tones, meaning partial hydrolysis; and c) without color change, indicating there was no hydrolysis.

Meanwhile, *T. hirsuta* RT-1 amylolytic capacity was tested following the same procedure described above. The tubes were inoculated with a 6 mm plug from a 5-day old PDA culture and incubated for seven days at 32 ± 2°C. Starch hydrolysis was evaluated with Lugol´s solution as described above.

2.3.2 Temperature tolerance

Yeast isolates thermotolerance was evaluated in YPD broth incubated at 32, 37, 40, 42, and 45°C, as describe by Kurtzman *et al* (2011). Growth was evaluated by turbidity.

For the *T. hirsuta* RT-1 thermotolerance assay, a 1 cm diameter plug (obtained from a 5-day PDA culture incubated at 32 ± 2°C), was inoculated in the center of RSF agar plates (15 g RSF; 20 g of agar for one liter of the medium) and incubated at 32, 35, 38 and 41°C for seven days. Growth was assessed by measuring the diameter (crosswise) of the colony with a digital vernier.

2.3.3 Ethanol tolerance

The yeast ethanol tolerance was determined in accordance with Lachance (1995), using a liquid medium. Aliquots of cell suspension of each isolate yeast (0.5 mL) were inoculated in 5 mL YM broth (BD Difco) supplemented with glucose at 8% (w/v) and absolute ethanol (3-12% (v/v) with serial increments of 1%. Then they were incubated at 30°C for 24 h. The growth was determined by turbidity.

T. hisuta RT-1 ethanol tolerance was tested as described above but on YM agar plates, instead of YM broth, supplemented with 8% (w/v) glucose. Warm ethanol at 3% (v/v) was added to the agar plate and then a plug of 1 cm diameter of a 5-day old PDA culture with the fungus was inoculated and incubated at 32 ± 2°C for 9 days. The procedure was repeated with subsequent 1% serial ethanol concentration increments, until no mycelial growth was observed.

2.4 Kinetics of direct reducing sugars release by T. hirsuta RT-1

A suspension of RSF at 14% (w/v) was prepared in a sterilized Erlenmeyer flask, then it was inoculated with two plugs of 1 cm diameter from a 5-day old PDA with *T. hirsuta* RT-1 culture. The flask was incubated for 14 days at 32 ± 2º C. An aliquot of 1 mL was sampled every two days from day 0 to day 14, to evaluate starch hydrolysis by quantifying the glucose released using the reducing sugar method proposed by Miller (1959). The samples were also used to quantify the α-amylase and laccase activity.

2.4.1 α- amylase activity of T. hirsuta RT-1

α-amylase activity was evaluated following the methodology in Ahmed et al (2018), with modifications. The analysis was conducted by mixing soluble starch at 1% (w/v) and the sample obtained from section 2.4 in a proportion of 1:1. The mix was diluted using a sodium acetate buffer (0.1 M, pH 5.0). The mix solution was incubated at 40°C for 20 min, then, it was cooled down to 4°C to stop the reaction. Enzyme activity was quantified by measuring the total reducing sugars released from starch hydrolysis with the Miller DNS procedure (1959). The increment in reducing sugar content indicates that the starch was hydrolyzed by *T. hirsuta* RT-1.

2.4.2 Laccase activity of T. hirsuta RT-1

Laccase activity was evaluated using the method described by Johannes and Majcherczyk (2000). The oxidation of ABTS was measured at 420 nm absorbance. The amount of enzyme was expressed as U/mL, one enzyme unit (U) being defined as the

amount of enzyme required to oxidize 1μmol of ABTS per min under assay conditions.

2.5 *Bio-saccharification and fermentation strategy*

Bio-saccharification procedure was performed in 250 mL Erlenmeyer flasks containing 100 mL of RSF sterile suspension at 14% (w/v) inoculated with two 1 cm-diameter plugs obtained from a 5-day old PDA with *T. hirsuta* RT-1 culture. The flasks were incubated in static conditions at 32 ± 2°C for 8 days. Then the fungal mycelium was removed manually, and the flasks were subsequently shaken at 150 rpm for 24 h in a Scorpion Scientific incubator (Model A52101A0, Mexico). A 1 mL sample was taken to determine glucose concentration by HPLC analysis.

Fermentation was induced by adding 1 mL of the *C. tropiclais* PL-1 inoculum (1 x 10^6 cells/mL) to each flask. The assessment of cell viability was conducted by measuring the quantity of cells using a Neubauer chamber, while discrimination between living and dead cells was facilitated using methyl blue. The flasks with the sample and the yeast inoculum were incubated under static semi-anaerobic conditions, at 32 ± 2°C for six days. Throughout the process, aliquots were taken daily to determine glucose consumption by HPLC analysis (described in analytical methods). The fermentation product was centrifuged at 4000 rpm for 20 min to remove solids, then 25 mL of the supernatant was diluted with 25 mL of water and distilled at 100°C until 25 mL of distillate were recovered. Ethanol concentration was quantified using a Gas Chromatograph (described below).

2.6 *Analytical methods*

Glucose analysis by HPLC (Agilent 1260 Infinity II Manual Injector, Santa Clara, USA), was performed using a Zorbax carbohydrate column 4.6 x 250 mm x 5μ (Agilent). The mobile phase was composed of acetonitrile and water (in a ratio of 75:25), and the flow rate was 2 mL/min at 40°C. The detector (RI) was at 35 °C. The injection volume was 20 μL. Glucose standard (CAS 50-99-7, Chem Service Inc., West Chester, PA, USA), was employed to perform a calibration curve.

Ethanol concentration was analyzed using Gas Chromatography (Perkin Elmer Clarus 500 model, Waltham, MA, USA) with a flame ionization detector (FID) and an EC-WAX 30 m x 32 mm x 0.25 μm column (Alltech Grace, Columbia, MA, USA). The carrier gas was nitrogen (N_2) at 7 psi and a 20 mL/min split; the injection temperature was of 120°C and 200°C for FID; and sample volume was of 2 μL.

2.7 *Fermentation conversion yield*

Theoretically 1 g starch equals 1.11 g glucose. Considering 0.511 as the conversion factor of glucose to ethanol, the theoretical yield of ethanol (Gronchi *et al*., 2019) is calculated as follows:

$$Theoretical\ ethanol\ yield\ (g) = \frac{(g\ starch \times 1.11\ glucose)}{1\ g\ starch} \times 0.511 \quad (1)$$

Fermentation efficiency was calculated following this equation:

$$Fermentation\ efficiency\ \% = \frac{ethanol\ produced\ (\frac{g}{l})}{theoretical\ ethanol\ yield\ (g/l)} \times 100 \quad (2)$$

3 Results and discussion

3.1 *Physiological characterization, molecular identification and phylogenetic analysis of the yeast isolate*

The identification of the four native yeasts, *C. tropicalis* PL-1, *P. kudriavzevii* TL-1, *H. guilliermondii* TL-2 and *M. caribbica* RSL-3, was corroborated by base pair sequence analysis of the D1/D2 Domain LSU 26S rDNA. The consensus sequences were aligned against the GenBank database and registered with accession numbers OM743847 (PL-1), OM743848 (TL-1), OM743849 (TL-2), and OM743850 (RSL-3). The native fungal strain RT-1 was identified as *Trametes hirsuta* by sequence of the ITS-5.8S region, and its GenBank accession number is OM732416.

Based on the D1/D2 Domain LSU 26S rDNA sequences of the native yeasts, their phylogenetic relationships were inferred by constructing a tree using the Neighbor-joining method (Figure 1). The four yeasts isolates are grouped into three clades (I, II and III) depending on their genetic relations.

Isolate PL-1 clustered with *Candida tropicalis* sequences; RSL-4 grouped with *Meyerozyma caribbica*; TL-2 with *Pichia kudriavzevii* and finally TL-3 with registered sequences in the GeneBank of *Hanseniaspora guillermondii*. It is worth mentioning that the isolates PL-1 and RSL-4 are the closest in terms of genetics, since they share clade I, which in turn is divided into two subclades (Ia and Ib) with 100% branch support. On the other hand, *P. kudriavzevii* (TL-2), is grouped far from the yeasts previously mentioned, and from *H. guilliermondii* (TL-3), in clade III. This last one is very distant genetically from the rest of the yeasts analyzed. The high support of each clade mentioned confirms their genetic relations.

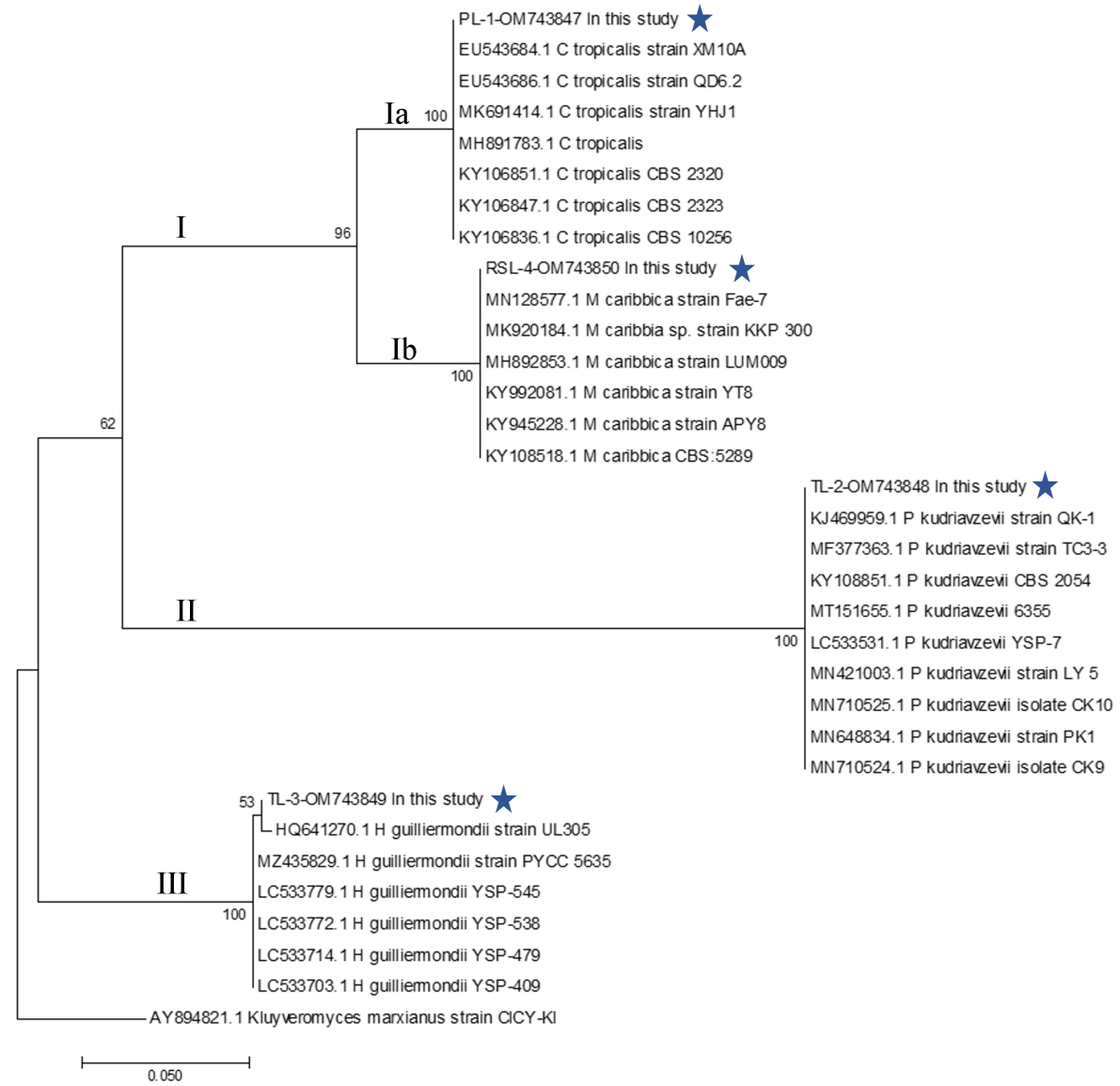

Fig. 1 Phylogenetic tree based on Neighbor Joining method analysis of the 26S rDNA D1/D2 domain of all yeast strains studied. GenBank accession numbers are indicated. The number in each node indicates the boostrap support. *Kluyveromyces maxianus* was the out-group.

Although all yeasts were isolated from ramon fruit, great genetic diversity can be observed among them. For example, *P. kudriavzevii* is a typical endophytic yeast associated with fruits (Matos *et al*., 2021). It has been associated with blossom and ripened apples, pears, and plums in southwest Slovakia (Vadkertiová *et al*., 2012). It is also have been isolated from a variety of niches such as soil samples from fruit gardens, agricultural farms and sugarcane factories (Phong *et al*., 2019). It is known as a typically thermal and furfural-tolerant yeast (Kurtzman *et al*., 2011).

Candida spp. is an indigenous yeast that is commonly found in natural or deliberate alcoholic fermentation. It has the ability to tolerate high concentrations of alcohol (Koulougliotis & Eriotou, 2016). *Candida tropicalis* has been isolated from different sources, including the primary juices of a distillery in Cuba, soil samples from a zoo's gardens in India (Mattam *et al*., 2016), and Moroccan olive-mill wastewater and traditional bread dough (Jamai *et al*., 2001).

Meyerozyma caribbica, was described as *Pichia caribbica*, to distinguish from *P. guilliermondii* (Vaughan-Martini *et al*., 2005). The yeasts isolated from sugar cane in Cuba, was named *Caribbica*. Further analysis repositioned it in a new genus, currently named *Meyerozyma* (Kurtzman & Suzuki, 2010). *M. caribbica* was also reported as a prevalent species in the natural wet fermentation of coffee fruits and beans in Brazil (Evangelista *et al*., 2015) and

Table 1. GeneBank accession numbers for the yeast isolates from *B. alicastrum*.

Isolate code	Accession numbers GeneBank	Species	Closed relative by BLAST	Sequence similarity similarity
PL-1	OM743847.1	*Candida tropicalis*	KY106836.1*(C. tropicalis)*	100 %
TL-2	OM743848.1	*Pichia kudriavzevii*	KY108851.1 *(P. kudriavzevii)*	100 %
TL-3	OM743849.1	*Hanseniaspora guilliermondii*	HQ641270.1*(H. guilliermondii)*	99 %
RSL-4	OM743850.1	*Meyerozyma caribbica*	KY108518.1*(M. caribbica)*	100 %

in *Mangifera indica* in Mexico (Bautista-Rosales *et al*., 2011). This species is therefore found worldwide and in cosmopolitan habit (Matos *et al*., 2021). Additionally, *M. caribbica* is known as a non-toxic yeast that has been used to produce the alcoholic beverage tequila in Mexico (Saucedo-Luna *et al*., 2011).

H. guilliermondii has been isolated from Douro grape musts (Albergaria *et al*., 2003). In other study, grape must, beer wort, grapes skin, and water-cured olives were processed to isolate *H. guillermondii*, a yeast specie which is added to enhance the aromatic profile of beer in mixed-culture fermentation with *Saccharomyces cerevisiae* (Bourbon-Melo *et al*., 2021).

The GenBank accession numbers of each sequence are listed in Table 1. These rDNA sequences constitute the first record of yeasts isolated from the fruit of the *Brosimun alicastrum* tree with the ability to hydrolyze starch.

3.2 Amylolytic capacity

The amylolytic capacity evaluated showed that all yeasts reacted as the control, and only the test tube containing *Candida tropicalis* (PL-1) exhibited a violet color indicating partial breakdown of starch (Figure 2). This confirms that *C. tropicalis* PL-1 has the capacity to synthesize glucoamylase in order to hydrolyze starch. Jamai *et al*. (2007) reported that *C. tropicalis* is a potentially useful organism for the commercial production of ethanol as it is capable of fermenting starch at a low rate (Cholis & Chanson, 2019). In this respect, *Candida* has been reported among the best producers of glucoamylases (α-1, 4-glucan glucohydrolase E.C. (3.2.1.3)), an enzyme that hydrolyzes α-1,4 glycosidic bonds from the non-reducing ends of starch, resulting in the production of glucose which can be further fermented to ethanol (Hostinová & Gašperík, 2010).

Filamentous fungi have been used as the source of glucoamylases for industrial purposes (Hostinová & Gašperík, 2010). In particular, basidiomycete fungi, such as *T. hirsuta*, synthetize α-amylase and glucoamylase (Jamai *et al*., 2007). Thus, it can directly ferment starch, wheat bran and rice straw to ethanol without acid or enzymatic hydrolysis (Okamoto *et al*., 2011). Olguin-Maciel *et al*. (2019), reported *T. hirsuta* (Bm-2) use in production of bioethanol in a consolidated bioprocess. In this study *T. hirsuta* RT-1 showed amylolytic activity through changes in coloration during the time of analysis (Figure 3), which went from a blue-purple color to light purple on the 4th day. However, by the 7th day the coloration had disappeared, confirming the saccharification capacity of starch. This color variation is a consequence of a reduction in the starch chain length related with α-amylase action (Visvanathan *et al*., 2020).

The color of the starch-iodine complex depends on the size of the amylose chain, and is colorless with less than 20 glucose units, red-violet (DP 30-38), blue-violet (DP 39-46) or blue (DP $\geq$ 47) (Visvanathan *et al*., 2020). These results are in accordance with other reports on the amylolytic capacity of *T. hirsuta* Bm-2 (Carrillo-Nieves *et al*., 2020; Olguin-Maciel *et al*., 2019; Zhang *et al*., 2020).

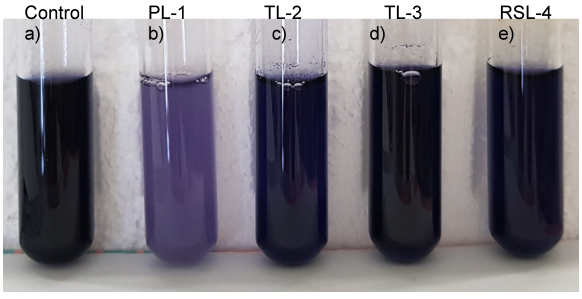

Fig. 2 Soluble starch hydrolysis of native yeasts strains: a) control; b) *Candida tropicalis* PL-1, c) *Pichia kudriavzevii* TL-2, d) *Hanseniaspora guilliermondii* TL-3 y e) *Meyerozyma caribbica* RSL-4.

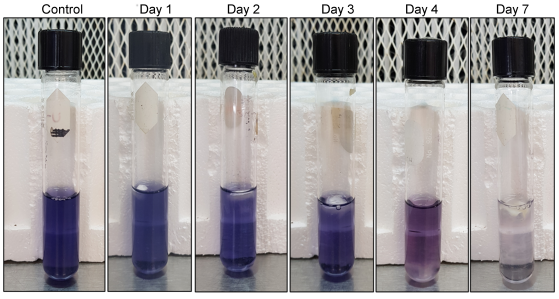

Fig. 3 Soluble starch hydrolysis of *T. hirsuta* RT-1.

3.3 Temperature tolerance

Use of thermotolerant yeasts to obtain ethanol offers several advantages, such as, high yields in

Table 2. Termotolerance of yeast strains to grow at different temperatures.

Yeast strains	32 °C	37 °C	40 °C	42 °C	45 °C
C. tropicalis PL-1	+ + +	+ + +	+ + +	+ + +	-
Pichia kudriavzevii TL-2	+ + +	+ + +	+ +	++	-
Hanseniaspora guilliermondii TL-3	+ + +	+ + +	+ +	-	-
Meyerozyma caribbica RSL-4	+ + +	+ +	-	-	-

+++good growth; ++ moderate growth; + weak growth; - no growth.

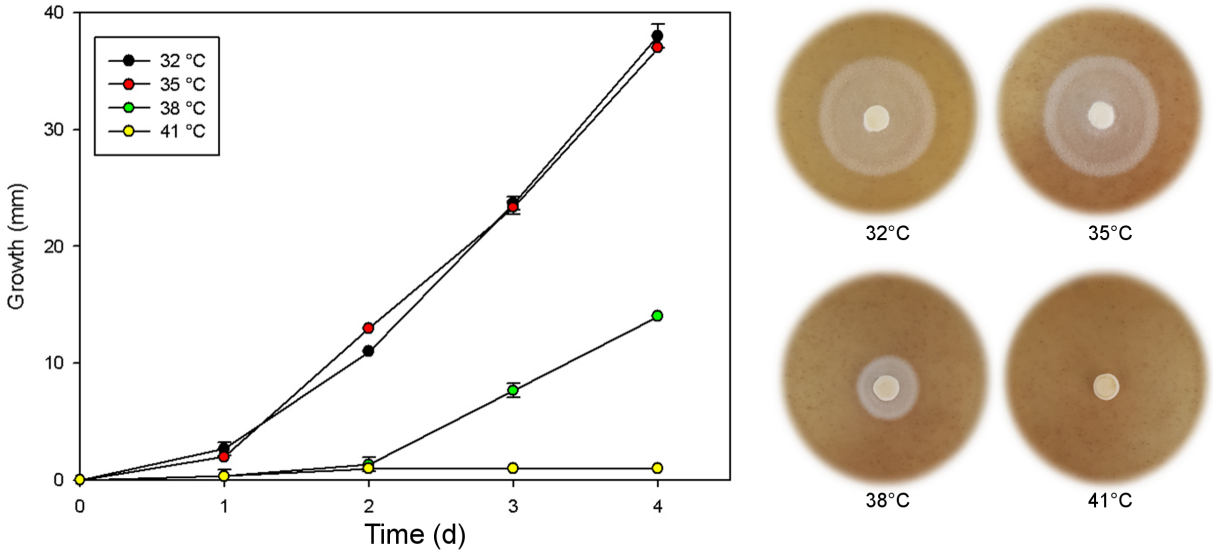

Fig. 4 *T. hirsuta* RT-1 strains tolerance to grow at different temperatures.

saccharification and fermentation products, decreased energy requirement for product recovery and a reduction in cooling costs (Arora *et al*., 2015). Therefore, the screening of yeasts that can sustain growth under a variety of inhibitory conditions e.g., high osmolality, high ethanol concentration, and/or elevated temperatures (>40°C) (Caspeta & Nielsen, 2015), is a priority. In this study, the isolates evaluated showed good growth at 32°C, although as temperature increased yeast resistance diminished. This is the case of *H. guilliermondii* TL-3 and *M. caribbica* RSL-4. *C. tropicalis* PL-1 and *Pichia kudriavzevii* TL-2 displayed good and moderate growth from 32°C up to 42°C (Table 2).

Candida species are considered strong candidates for heat and ethanol tolerance (Kadam & Schmidt, 1997). *C. tropicalis* in particular is useful for ethanol production from starch and lignocellulosic biomass through its cellulase activity (Cholis & Chanson, 2019; Tolieng *et al*., 2018). This species exhibits a lower glycolytic flux and higher oxygen consumption i.e. a Crabtree negative effect, compared with *S. cerevisiae* (Jamai *et al*., 2007). *C. tropicalis* tolerance to 42°C, has not been reported before. This tolerance is well-suited for simultaneous saccharification and fermentation of hemicellulose that occur at a temperature higher than 40°C (Jamai *et al*., 2001). On the other hand, *P. kudriavzevii* is well-known thermotolerant yeast specie that showed good growth at 42° C. Although the highest yields at temperatures over 40°C and low yield of ethanol at 28°C have been reported (Matos *et al*., 2021). In particular, the CM4.2 strain of *P. kudriavzevii* produced the highest ethanol concentration, volumetric ethanol productivity, and ethanol yield with a relatively high fermentation efficiency at 37, 40, and 45°C compared to *Saccharomyces cerevisiae* (Phong *et al*., 2019). *M. caribbica* RSL-4 does not tolerate high temperatures. However, others such as CC003 of *M. caribbica* is a promissing fermenter for alcoholic beverages. Although not tolerant to high temperatures, it is osmotolerant and exhibits high ethanol yield (Matos *et al*., 2021). MJTm3 of *M. caribbica* demonstrated remarkable stress tolerance and fermentative activity in sugarcane molasses at 45°C (Hawaz *et al*., 2023). As for *H. guillermondi*, there was not reported tolerance to heat.

In a consolidated bioprocess, thermophilic enzymes and yeasts are often added at the same time to ensure simultaneous saccharification and fermentation of cellulosic biomass to ethanol. Reducing the use of saccharifying enzymes is essential to lowering the process costs. (Tanimura *et al*., 2015).

Table 3. Yeast isolates growing at different ethanol concentrations.

Yeast	Ethanol concentrations (%)					
	3-4	5-6	7-8	9	10-11	12
Candida tropicalis PL-1	+ + +	+ +	+	-	-	-
Pichia kudriavzevii TL-2	+ + +	+ +	+ +	+ +	+	-
Hanseniaspora guilliermondii TL-3	+ + +	+ +	+ +	+ +	+	-
Meyerozyma caribbica RSL-4	+ + +	+ +	+ +	+ +	+	-

+++good growth; ++ moderate growth; + weak growth; - no growth.

Similarly, the fungus was evaluated to determinate its thermotolerance. *Trametes hirsuta*, produces laccase, a multicopper enzyme with different biological functions and biotechnological applications (Zapata-Castillo *et al*., 2015). The temperature of incubation (around 35-40°C) plays a key role in laccase, lignin peroxidase and Mn-peroxidase production (Krumova *et al*., 2018).

Specifically, *T. hirsuta* grows as temperature is increased. Although it has been reported that it can grow from 12-42°C, its maximum biomass production was achieved at 35°C (Krumova *et al*., 2018). In this study *T. hirsuta* RT-1 showed an optimum growth at 32°C. Temperatures higher than this cause mycelium development to start to diminish and disappear as temperatures increase (Figure 4). Different species from the *Trametes* genus have shown optimal growth at temperatures between 30 and 40°C (Magan, 2008). That is why the optimum temperature for *T. hirsuta* RT-1 growth was chosen to carry out further experiments and the sequential saccharification and fermentation.

3.4 Ethanol tolerance

Ethanol accumulation in the culture broth could affect yeast performance. The yeasts species tolerance to growth in different ethanol concentrations indicated all yeasts enjoy good growth at 3-4 % (v/v) of ethanol (Table 3). However, as ethanol concentration increased, the growth began to diminish.

As mentioned above, all the native yeasts isolated in this study showed good growth at 3-4% ethanol (v/v). However, as ethanol concentrations increased the growth began to diminish from moderate, to weak, and then negative.

In the case of PL-1 isolates, growth ceased at concentrations of 7-8 % (v/v) of ethanol. For the rest of the yeasts, growth ceased at concentrations of 10-11% of ethanol (v/v).

Each yeast species has a different capacity to tolerate, grow in and/or survive in the presence of ethanol (Snoek *et al*., 2016). It has been reported that *C. tropicalis* KPC isolated from sugarcane field soil in Thailand, and *C. tropicalis* KKU-105, isolated from northern Thailand, can tolerate ethanol concentrations up to 20% and 10% (v/v), respectively (Pongcharoen & Kawano-Kawada, 2018), which contrast with the 8% (v/v) tolerated by *C. tropicalis* PL-1.

The yeast isolates *P. kudriavzevii* TL-2, *M. caribbica* RSL-4 and *H. guilliermondii* TL-3 tolerated 11% (v/v) ethanol. However, there are reports in the literature of several strains of the above-mentioned species with a higher ethanol tolerance. In 2017, Techaparin *et al*. (2017) reported that the strains KKUTH33 and KKUTH43 isolated from the northeastern region in Thailand tolerated up to 13% (v/v) ethanol, the same tolerance was reported by Pongcharoen (2022).

The fungus *T. hirsuta* RT-1 was expected to have a similar ethanol tolerance to that reported by Olguin-Maciel *et al*. (2019) for *T. hirsuta* Bm-2, which exhibited a vigorous growth at 10% (v/v) ethanol and fermentative capacity, due to the capacity of some basidiomycetes to synthesize the alcohol dehydrogenase enzyme. However, the RT- 1 strain presented a low ethanol tolerance as the mycelium growth was minimal at 3% (v/v). For this reason no further experiments were done. A similar behavior was seen by Paschos (2015), who observed that biomass and growth rate of *F. oxysporum* in aerobic conditions diminished in the presence of ethanol (1-6% w/v). The results of this experiment implied that the RT-1 isolate will only take part in the saccharification process of the RSF and that the fermentation would be carried out by the PL-1 isolate, which was chosen through the tests of characterization, principally the amylolytic capacity and ethanol tolerance.

3.5 Direct reducing sugars release kinetics, α- amylase and laccase activity of T. hirsuta RT-1

The amylolytic capacity of *T. hirsuta* RT-1 was measured and is shown in Figure 5; the highest concentration of reducing sugars (RS) was 28.95 ± 2.17 g/L and was recorded on day 9. This result is comparable to the one reported by Olguín *et al*. (2019) with *T. hirsuta* Bm-2 strain, which was 30 g/L on day 8. The α-amylase activity was already detectable before inoculating *T. hirsuta* RT-1 as seeds have enzymes like α or β–amylases. After the addition of fungus the activity increased due to the induction of the enzyme by starch or its hydrolytic products such as maltose, which are present in the RSF (Gupta *et al*., 2003; Saranraj & Stella, 2013).

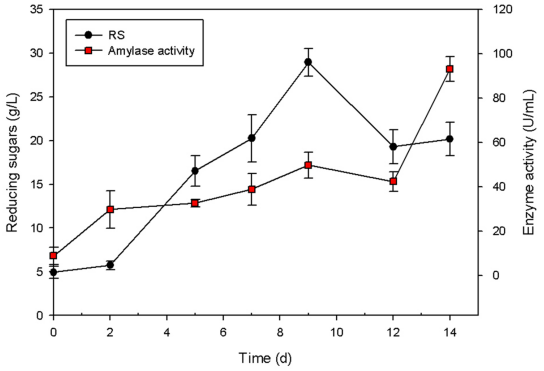

Fig. 5 Direct reducing sugars release kinetics and α-amylase activity of *T. hirsuta* RT-1 strain.

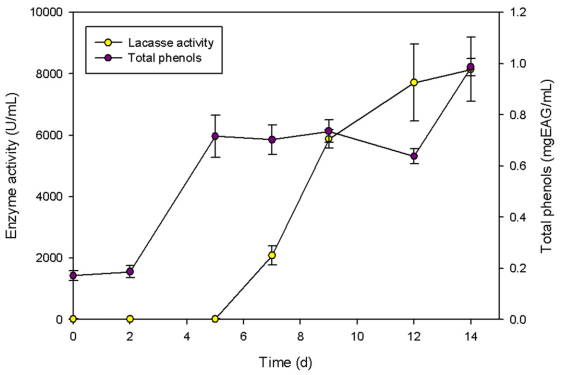

Fig. 6. Lacasse activity of *T. hirsuta* RT-1 and total phenols concentration.

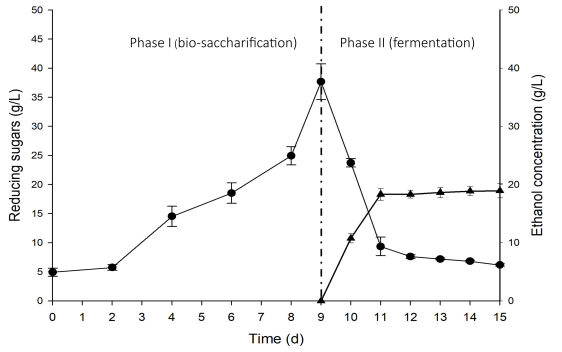

Fig. 7 Consolidated bio-saccharification and fermentation of ramon seed flour (RSF) by *T. hirsuta* RT-1 and *C. tropiccalis* (PL-1) strains.

The production of α-amylase agrees with Pervez *et al*. (2014), who mentioned that amylase from fungal sources was normally produced after 3 to7 days of incubation. The first major activity of the enzyme was on day 9, with 49.77 ± 7.54 U/mL. The increase in reducing sugars (RS) was simultaneous. After that, the activity dropped, probably due to the glucose concentration, because it functions as a catabolic repressor for the enzyme synthesis and once the fungus had consumed the glucose, the enzyme activity increased up to 93.15 ± 6.35 U/mL. In comparison to the result of 135 U/mL reported by Olguin *et al*. (2019) for Bm-2 isolate in the same substrate, RT-1 displayed less enzyme activity, establishing a metabolic difference within the same species. This contrast between the strains occurs as a result of variations in the production and specificity of the enzymes towards the starch, amylose and amylopectin of each microorganism, even among the same genera and species of isolate origin (Gopinath *et al*., 2017; Gupta *et al*., 2003).

The laccase enzyme is a multicopper oxidases which plays an important role in the degradation of lignin (Shraddha *et al*., 2011), and thus it can participate in saccharification by depolymerization of lignin or fibroproteic matrix, making starch granules of RSF susceptible to α-amylase. The maximum laccase activity of 8,920 ± 1,236 U/mL was achieved on day 14th (Figure 6) and was associated with diminishing total phenols. The phenolic compounds in RSF are found as gallic acid, ρ-hydroxybenzoic acid, vanillic and caffeic acid, ρ-coumaric acid and chlorogenic acid. Similary to RT-1, *T. hirsuta* Bm-2 laccase enzyme activity was induced in the presence of vanillic acid (Tapia-Tussell *et al*., 2015). To explain the laccase action, it was assumed that hydrolytic enzymes of proteins, lipids and carbohydrates produced by *T. hirsuta* RT-1 release the phenolic compounds since they are also associated with simple or complex carbohydrates, lipids and fiber (Quirós-Sauceda *et al*., 2014).

3.6 Bio-saccharification and fermentation strategy

As shown in figure 7, the biosaccharification and fermentation process of RSF results in an increase in reducing sugars content (35 ± 3.32 g/L) until the 9th day; additionally, the reduction in the viscosity of the biomass was observed. This is a strong indicator of that *T. hirsuta* RT-1 was producing a mixture of α-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3), two enzymes responsible for hydrolysis of starch into glucose units (Cripwell *et al*., 2020).

It is worth mentioning that the glucose concentration increased from 24.89 ± 0.81 g/L under static conditions to 37.78 ± 1.24 g/L after agitation. This increment is probably due to hydrolysis of RSF by *T. hirsuta* RT-1. This effect on glucose concentration might be due to the low agitation speed, which allowed the production and activity of amylases that improved enzyme homogenization, substrate-enzyme interactions, and distribution of nutrients and oxygen within the bioreactor (Henshaw & Wakil, 2019; Roldan-Cruz *et al*., 2021). This increment in glucose concentration after agitation had already been reported by Farid *et al*. (2002). At 200-300 rpm the greatest quantity of glucose was produced, and at 150-200 rpm, α-amylase and glucoamylase enzymes. The

effects of mechanical agitation on α-amylase enzyme activity, are not fully understood. It has already been reported that agitation at high speed denaturalizes the enzyme structure, affecting its activity (Henshaw & Wakil, 2019; Roldan-Cruz *et al*., 2021; Rousset & Schlich, 1989; Sivaramakrishnan *et al*., 2006). Another factor to consider is that optimum fungal growth conditions must be similar to those of amylase production, in order to obtain the maximum yield (Rousset & Schlich, 1989).

At the end of saccharification by *T. hirsuta* RT-1, *C. tropicalis* PL-1 was added to the same flask for the start-up of the fermentation phase, with a glucose concentration of 39 ± 3.27 g/L. The sugar intake and ethanol production (Figure 7, phase II), indicated that *C. tropicalis* PL-1, consumed 93% of glucose and produced 18.93 ± 1.78 g/L of ethanol. Thus, the fermentation efficiency was of 95 %. The initial and final pH were 5.45 and 4.60, respectively.

It was observed that the highest glucose consumption occured during the first two days, and the highest ethanol production was also detected at the same time. On the 3rd day, glucose consumption and ethanol production reached a plateau.

In this context, the ideal fermentation time is between 48 and 72 h, since the ethanol production decreases as the fermentation time increases from 72 h to 96 h (Zabed *et al*., 2017).

It is possible that the viability of *T. hirsuta* RT-1, was affected by its low ethanol tolerance. Although more studies are needed to determinate its effect on fungus during the fermentation process, the mycelium it is a suitable source of nutrients (proteins and polysaccharides) for yeast. Romano *et al*. (2006) reported that some species of *Candida* presented proteolytic, glycosidic and pectinolytic activity, which should be evaluated in *C. tropicalis* PL-1.

In the sequential biosaccharification and fermentation strategy, 18.93 ± 1.78 g/L of ethanol was produced, which is less than the 37.76g/L that was reported by Olguin *et al*. (2019) for RSF using a separate hydrolysis and fermentation with *C. tropicalis* PL-1. However, the ethanol production in this study was higher than the ethanol concentration reported by the same authors (13g/L) in a consolidated bioprocess (CBP) with the same raw material and the native isolate *T. hirsuta* Bm-2.

The simultaneous biosaccharification and fermentation process is a suitable solution for CBP drawbacks, by finding the optimal conditions within a single reactor for hydrolytic enzyme secretion, saccharification, and fermentation. The low biosaccharification rate may affected the sequential process for ethanol production, and improvements in fermentation will be required, considering that it can be affected due multiple factors, such as heat and ethanol stress, nutrient deficiency, sugar concentration, and viscosity in the medium, among others.

Some benefits of this strategy are the direct production of amylase enzymes by the fungus RT-1, which allows for hydrolysis without using commercial enzymes. This leads to a reduction in the ethanol production costs, as well as a reduction in the probability of contamination, since the entire process is carried out in a single reactor. On the other hand, as part of the search for a sustainable process, a value-added compound is obtained, namely a fungal biomass with high protein content, that can be used as a food supplement for animals.

Conclusions

The identification of native microorganisms with biotechnological potential can help in the production of second-generation biofuels from starchy biomass due to their natural adaptation to the raw material, which could improve the yields of liquefaction, hydrolysis and fermentation, thus making the process more profitable. Their genetic and biochemical identification allowed for the proper selection of the most suitable biological specimens for bio saccharification and sequential fermentation. The organisms selected for the CBS were the basidiomycete *T. hirsuta* RT-1 and the yeast *C. tropicalis* PL-1. The fungus displayed low ethanol tolerance, which restricted its role to the bio saccharification phase. While PL- 1 was used for fermentation, reaching a conversion of 95% of the fermentable sugars into bioethanol, bioethanol production in the CBS was 18.93 ± 1.78 g/L. It is important to point out that the process was carried out in a single reactor and no reagents or commercial enzymes were used, which could make bioethanol production more sustainable. Further studies can be focused on the enhancement of saccharification and fermentation, on the identification of extracellular enzymes, and on the interaction of the fungus (*T. hirsuta* RT-1) with the yeast (*C. tropicalis* PL-1). During the CBS, in addition to amylase production, starch hydrolysis and fermentation, a laccase enzyme was obtained that can help in the bioconversion of lignocellulosic biomass, making the entire process a novel opportunity to develop a biorefinery approach for the exploitation of ramon seed and other feedstocks.

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References

Ahmed, S. A., Mostafa, F. A., & Ouis, M. A. (2018). Enhancement stability and catalytic activity of immobilized α-amylase using bioactive phospho-silicate glass as a novel inorganic support. *International Journal of Biological Macromolecules*, 112, 371–382.

Albergaria, H., Torrão, A. R., Hogg, T., & Gírio, F. M. (2003). Physiological behaviour of *Hanseniaspora guilliermondii* in aerobic glucose-limited continuous cultures. *FEMS Yeast Research*, 3(2), 211–216.

Arora, R., Behera, S., & Kumar, S. (2015). Bioprospecting thermophilic/thermotolerant microbes for production of lignocellulosic ethanol: a future perspective. *Renewable and Sustainable Energy Reviews*, 51, 699–717.

Barnett, J. A., Payne, R. W., & Yarrow, D. (1990). Yeasts: characteristics and identification.

Bautista-Rosales, P. U., Servín-Villegas, R., Calderón-Santoyo, M., & Ragazzo-Sánchez, J. A. (2011). Control biológico de *Colletotrichum* sp. utilizando levaduras antagonistas nativas del mango. *3er Congreso Internacional de Biología, Química y Agronomía. Universidad Autónoma de Guadalajara*, AC, Zapopan, Jalisco, México, 1–10.

Bourbon-Melo, N., Palma, M., Rocha, M. P., Ferreira, A., Bronze, M. R., Elias, H., & Sá-Correia, I. (2021). Use of *Hanseniaspora guilliermondii* and *Hanseniaspora opuntiae* to enhance the aromatic profile of beer in mixed-culture fermentation with *Saccharomyces cerevisiae*. *Food Microbiology*, 95, 103678.

Bušić, A., Mardetko, N., Kundas, S., Morzak, G., Belskaya, H., Ivčanić šantek, M., Komes, D., Novak, S., & šantek, B. (2018). Bioethanol production from renewable raw materials and its separation and purification: a review. *Food Technology and Biotechnology*, 56(3), 289–311.

Carrillo-Nieves, D., Saldarriaga-Hernandez, S., Gutiérrez-Soto, G., Rostro-Alanis, M., Hernández-Luna, C., Alvarez, A. J., Iqbal, H. M. N., & Parra-Saldívar, R. (2020). Biotransformation of agro-industrial waste to produce lignocellulolytic enzymes and bioethanol with a zero waste. *Biomass Conversion and Biorefinery*, 1–12.

Caspeta, L., & Nielsen, J. (2015). Thermotolerant yeast strains adapted by laboratory evolution show trade-off at ancestral temperatures and preadaptation to other stresses. *MBio*, 6(4), 10–1128.

Cholis, M., & Chanson, C. (2019). Molecular identification and potential ethanol production of long-term thermo-tolerant yeast *Candida Tropicalis*. *IOP Conference Series: Earth and Environmental Science*, 239(1), 12004.

Cripwell, R. A., Favaro, L., Viljoen-Bloom, M., & van Zyl, W. H. (2020). Consolidated bioprocessing of raw starch to ethanol by *Saccharomyces cerevisiae*: Achievements and challenges. *Biotechnology Advances*, 42, 107579.

Evangelista, S. R., Miguel, M. G. da C. P., Silva, C. F., Pinheiro, A. C. M., & Schwan, R. F. (2015). Microbiological diversity associated with the spontaneous wet method of coffee fermentation. *International Journal of Food Microbiology*, 210, 102–112.

Farid, M. A., El-Enshasy, H. A., & Noor El-Deen, A. M. (2002). Alcohol production from starch by mixed cultures of *Aspergillus awamori* and immobilized *Saccharomyces cerevisiae* at different agitation speeds. *Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms*, 42(3), 162–171.

Favaro, L., Jansen, T., & van Zyl, W. H. (2019). Exploring industrial and natural *Saccharomyces cerevisiae* strains for the bio-based economy from biomass: the case of bioethanol. *Critical Reviews in Biotechnology*, 39(6), 800–816.

Ferreira, J. A., Agnihotri, S., & Taherzadeh, M. J. (2019). Waste biorefinery. In *Sustainable resource recovery and zero waste approaches* (pp. 35–52). Elsevier.

Gopinath, S. C. B., Anbu, P., Arshad, M. K. M., Lakshmipriya, T., Voon, C. H., Hashim, U., & Chinni, S. V. (2017). Biotechnological processes in microbial amylase production. *BioMed Research International*, 2017(1), 1272193.

Gronchi, N., Favaro, L., Cagnin, L., Brojanigo, S., Pizzocchero, V., Basaglia, M., & Casella, S. (2019). Novel yeast strains for the efficient saccharification and fermentation of starchy by-products to bioethanol. *Energies*, 12(4), 714.

Gupta, R., Gigras, P., Mohapatra, H., Goswami, V. K., & Chauhan, B. (2003). Microbial α-amylases: a biotechnological perspective. *Process Biochemistry*, 38(11), 1599–1616.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41(41), 95–98.

Hawaz, E., Tafesse, M., Tesfaye, A., Kiros, S., Beyene, D., Kebede, G., Boekhout, T., Groenwald, M., Theelen, B., & Degefe, A. (2023). Optimization of bioethanol production from sugarcane molasses by the response surface methodology using *Meyerozyma caribbica* isolate MJTm3. *Annals of Microbiology*, 73(1), 2.

Henshaw, E., & Wakil, S. M. (2019). Effect of agitation speed and incubation time on amylase production by *Bacillus* species isolated from malted and fermented Maize (*Zea mays*). *Microbiology Research Journal International*, 27(3), 1–7.

Hernández-González, O., Vergara-Yoisura, S., & Larqué-Saavedra, A. (2014). Studies on the productivity of *Brosimum alicastrum* a tropical tree used for animal feed in the Yucatan Peninsula. *Bothalia Journal*, 44(6), 70–81.

Hostinová, E., & Gašperík, J. (2010). Yeast glucoamylases: molecular-genetic and structural characterization. *Biologia*, 65, 559–568.

Huchin Poot, E. G. (2015). Aislamiento de la microbiota del fruto de *Brosimum alicastrum* swartz para su uso en la producción de bioetanol [Tesis de maestría, Centro de Investigación Científica de Yucatán]. Repositorio académico CICY.

Jahangeer, M., Rehman, M. U., Nelofer, R., Nadeem, M., Munir, B., Smułek, W., Jesionowski, T., & Qamar, S. A. (2024). Biotransformation of lignocellulosic biomass to value-added bioproducts: Insights into bio-saccharification strategies and potential concerns. *Topics in catalysis*, 1-22.

Jamai, L., Ettayebi, K., El Yamani, J., & Ettayebi, M. (2007). Production of ethanol from starch by free and immobilized *Candida tropicalis* in the presence of α-amylase. *Bioresource Technology*, 98(14), 2765–2770.

Jamai, L., Sendide, K., Ettayebi, K., Errachidi, F., Hamdouni-Alami, O., Tahri-Jouti, M. A., McDermott, T., & Ettayebi, M. (2001). Physiological difference during ethanol fermentation between calcium alginate-immobilized *Candida tropicalis* and *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, 204(2), 375–379.

Johannes, C., & Majcherczyk, A. (2000). Laccase activity tests and laccase inhibitors. *Journal of Biotechnology*, 78(2), 193–199.

Kadam, K. L., & Schmidt, S. L. (1997). Evaluation of *Candida acidothermophilum* in ethanol production from lignocellulosic biomass. *Applied Microbiology and Biotechnology*, 48, 709–713.

Kechkar, M., Sayed, W., Cabrol, A., Aziza, M., Ahmed Zaid, T., Amrane, A., & Djelal, H. (2019). Isolation and identification of yeast strains from sugarcane molasses, dates and figs for ethanol production under conditions simulating algal hydrolysate. *Brazilian Journal of Chemical Engineering*, 36(1), 157–169.

Koulougliotis, D., & Eriotou, E. (2016). Isolation and Identification of Endogenous Yeast Strains in Grapes and Must Solids of *Mavrodafni Kefalonias* and Antioxidant Activity of the Produced Red Wine. *Fermentation technology*, 5(1), 1-9.

Krumova, E., Kostadinova, N., Miteva-Staleva, J., Stoyancheva, G., Spassova, B., Abrashev, R., & Angelova, M. (2018). Potential of ligninolytic enzymatic complex produced by white-rot fungi from genus *Trametes* isolated from Bulgarian forest soil. *Engineering in Life Sciences*, 18(9), 692–701.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6), 1547–1549.

Kurtzman, C., Fell, J. W., & Boekhout, T. (2011). The yeasts: a taxonomic study. Elsevier.

Kurtzman, C. P., & Suzuki, M. (2010). Phylogenetic analysis of ascomycete yeasts that form coenzyme Q-9 and the proposal of the new genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, and *Scheffersomyces*. *Mycoscience*, 51(1), 2–14.

Lachance, M.-A. (1995). Yeast communities in a natural tequila fermentation. Antonie Van Leeuwenhoek, 68, 151–160.

Lennartsson, P. R., Erlandsson, P., & Taherzadeh, M. J. (2014). Integration of the first and second generation bioethanol processes and the importance of by-products. *Bioresource Technology*, 165, 3–8.

Losoya-Sifuentes, C., Pinto-Jimenez, K., Cruz, M., Rodriguez-Jasso, R. M., Ruiz, H. A., Loredo-Treviño, A., ... & Belmares, R. (2023). Determination of nutritional and antioxidant properties of Maya Nut flour (*Brosimum alicastrum*) for development of functional foods. *Foods*, 12(7), 1398.

Magan, N. (2008). Ecophysiology: impact of environment on growth, synthesis of compatible solutes and enzyme production. *British Mycological Society Symposia Series*, 28, 63–78.

Matos, Í. T. S. R., de Souza, V. A., D'Angelo, G. do R., Astolfi Filho, S., do Carmo, E. J., & Vital, M. J. S. (2021). Yeasts with Fermentative Potential Associated with Fruits of Camu-Camu (*Myrciaria dubia*, Kunth) from North of Brazilian Amazon. *The Scientific World Journal*, 2021(1), 9929059.

Mattam, A. J., Kuila, A., Suralikerimath, N., Choudary, N., Rao, P. V. C., & Velankar, H. R. (2016). Cellulolytic enzyme expression and simultaneous conversion of lignocellulosic sugars into ethanol and xylitol by a new *Candida tropicalis* strain. *Biotechnology for Biofuels*, 9, 1–12.

Meiners, M., Sánchez-Garduño, C., & De Blois, S. (2009). El ramón: Fruto de nuestra cultura y raíz para la conservación. *Biodiversitas*, 87, 7–10.

Miller, G. L. (1959). Modified DNS method for reducing sugars. *Analytical Chemistry*, 31(3), 426–428.

Okamoto, K., Nitta, Y., Maekawa, N., & Yanase, H. (2011). Direct ethanol production from starch, wheat bran and rice straw by the white rot fungus *Trametes hirsuta*. *Enzyme and Microbial Technology*, 48(3), 273–277.

Olguin-Maciel, E., Larqué-Saavedra, A., Lappe-Oliveras, P., Barahona-Pérez, L., Alzate-Gaviria, L., Chablé-Villacis, R., Domínguez-Maldonado, J., Pacheco-Catalán, D., Ruíz, H., & Tapia-Tussell, R. (2019). Consolidated Bioprocess for Bioethanol Production from Raw Flour of *Brosimum alicastrum* Seeds Using the Native Strain of *Trametes hirsuta* Bm-2. *Microorganism*, 7(483).

Olguin-Maciel, E., Larqué-Saavedra, A., Pérez-Brito, D., Barahona-Pérez, L. F., Alzate-Gaviria, L., Toledano-Thompson, T., Lappe-Oliveras, P. E., Huchin-Poot, E. G., & Tapia-Tussell, R. (2017). *Brosimum alicastrum* as a novel starch source for bioethanol production. *Energies*, 10(10), 1574.

Olguin-Maciel, E., Singh, A., Chable-Villacis, R., Tapia-Tussell, R., & Ruiz, H. A. (2020). Consolidated bioprocessing, an innovative strategy towards sustainability for biofuels production from crop residues: an overview. *Agronomy*, 10(11), 1834.

Ozer, H. K. (2017). Phenolic compositions and antioxidant activities of Maya nut (*Brosimum alicastrum*): Comparison with commercial nuts. *International Journal of Food Properties*, 20(11), 2772-2781.

Paschos, T., Xiros, C., & Christakopoulos, P. (2015). Ethanol effect on metabolic activity of the ethalogenic fungus *Fusarium oxysporum*. *BMC Biotechnology*, 15, 1–12.

Phong, H. X., Klanrit, P., Dung, N. T. P., Yamada, M., & Thanonkeo, P. (2019). Isolation and characterization of thermotolerant yeasts for the production of second-generation bioethanol. *Annals of Microbiology*, 69, 765–776.

Pongcharoen, P. (2022). The ability of *Pichia kudriavzevii* to tolerate multiple stresses makes it promising for developing improved bioethanol production processes. *Letters in Applied Microbiology*, 75(1), 36–44.

Pongcharoen, P., & Kawano-Kawada, M. (2018). Identification and characterization of *Candida tropicalis* isolated from soil of sugarcane plantation in Thailand for ethanol production. *Asia-Pacific Journal of Science Technology*, 23.

Quirós-Sauceda, A. E., Palafox-Carlos, H., Sáyago-Ayerdi, S. G., Ayala-Zavala, J. F., Bello-Perez, L. A., Alvarez-Parrilla, E., De La Rosa, L. A., González-Córdova, A. F., & González-Aguilar, G. A. (2014). Dietary fiber and phenolic compounds as functional ingredients: interaction and possible effect after ingestion. *Food & Function*, 5(6), 1063–1072.

Ramos-Villacob, V., Figueroa-Flórez, J. A., Salcedo-Mendoza, J. G., Hernandez-Ruydíaz, J. E., & Romero-Verbel, L. A. (2024) Development of modified cassava starches by ultrasound-assisted amylose/lauric acid complex formation. *Revista Mexicana de Ingeniería Química*, 23(1), 1-15.

Roldan-Cruz, C., Garcia-Hernandez, A., Alvarez-Ramirez, J., & Vernon-Carter, E. J. (2021). Effect of the stirring speed in the in vitro activity of α-amylase. *Food Hydrocolloids*, 110, 106127.

Romano, P., Capece, A., & Jespersen, L. (2006). Taxonomic and ecological diversity of food and

beverage yeasts. *In Yeasts in food and beverages* (pp. 13–53). Springer.

Rousset, S., & Schlich, P. (1989). Amylase production in submerged culture using principal component analysis. *Journal of Fermentation and Bioengineering*, 68(5), 339–343.

Saranraj, P., & Stella, D. (2013). Fungal amylase—a review. *International Journal of Microbiological Research*, 4(2), 203–211.

Satyanarayana, T. (2009). Yeast biotechnology: diversity and applications. Springer.

Saucedo-Luna, J., Castro-Montoya, A. J., Martinez-Pacheco, M. M., Sosa-Aguirre, C. R., & Campos-Garcia, J. (2011). Efficient chemical and enzymatic saccharification of the lignocellulosic residue from *Agave tequilana* bagasse to produce ethanol by *Pichia caribbica*. *Journal of Industrial Microbiology and Biotechnology*, 38(6), 725–732.

Shraddha, Shekher, R., Sehgal, S., Kamthania, M., & Kumar, A. (2011). Laccase: microbial sources, production, purification, and potential biotechnological applications. *Enzyme Research*, 2011(1), 217861.

Sivaramakrishnan, S., Gangadharan, D., Nampoothiri, K. M., Soccol, C. R., & Pandey, A. (2006). α-Amylases from Microbial Sources--An Overview on Recent Developments. *Food Technology & Biotechnology*, 44(2).

Snoek, T., Verstrepen, K. J., & Voordeckers, K. (2016). How do yeast cells become tolerant to high ethanol concentrations? *Current Genetics*, 62(3), 475–480.

Steensels, J., Snoek, T., Meersman, E., Nicolino, M. P., Voordeckers, K., & Verstrepen, K. J. (2014). Improving industrial yeast strains: exploiting natural and artificial diversity. *FEMS Microbiology Reviews*, 38(5), 947–995.

Suárez-Castillo, G.M., Salcedo-Guadalupe, J.G., Contreras-Lozano, K.P., Rangel-Pérez, M.G., Cervera-Ricardo, M.A., & Figueroa-Flórez, J.A. (2024). Increase in the degree of substitution of cassava starches by dual modification processes. *Revista Mexicana de Ingeniería Química*, 23(3), 1-15.

Tanimura, A., Kikukawa, M., Yamaguchi, S., Kishino, S., Ogawa, J., & Shima, J. (2015). Direct ethanol production from starch using a natural isolate, *Scheffersomyces shehatae*: toward consolidated bioprocessing. *Scientific Reports*, 5(1), 1–7.

Tapia-Tussell, R., Lappe, P., Ulloa, M., Quijano-Ramayo, A., Cáceres-Farfán, M., Larqué-Saavedra, A., & Perez-Brito, D. (2006). A rapid and simple method for DNA extraction from yeasts and fungi isolated from *Agave fourcroydes*. *Molecular Biotechnology*, 33, 67–70.

Tapia-Tussell, R., Pérez-Brito, D., Torres-Calzada, C., Cortés-Velázquez, A., Alzate-Gaviria, L., Chablé-Villacís, R., & Solís-Pereira, S. (2015). Laccase gene expression and vinasse biodegradation by *Trametes hirsuta* strain Bm-2. *Molecules*, 20(8), 15147–15157.

Techaparin, A., Thanonkeo, P., & Klanrit, P. (2017). High-temperature ethanol production using thermotolerant yeast newly isolated from Greater Mekong Subregion. *Brazilian Journal of Microbiology*, 48(3), 461–475.

Tolieng, V., Kunthiphun, S., Savarajara, A., & Tanasupawat, S. (2018). Diversity of yeasts and their ethanol production at high temperature. *Journal of Applied Pharmaceutical Science*, 8(2), 136–142.

Umeh, S. O., Agwuna, L. C., & Okafor, U. C. (2017). Yeasts from local sources: an alternative to the conventional brewer's yeast. *World Wide Journal of Multidisciplinary Research and Development,* 30, 191–195.

Vadkertiová, R., Molnárová, J., Vránová, D., & Sláviková, E. (2012). Yeasts and yeast-like organisms associated with fruits and blossoms of different fruit trees. *Canadian Journal of Microbiology*, 58(12), 1344–1352.

Vaughan-Martini, A., Kurtzman, C. P., Meyer, S. A., & O'Neill, E. B. (2005). Two new species in the *Pichia guilliermondii* clade: *Pichia caribbica* sp. nov., the ascosporic state of *Candida fermentati*, and *Candida carpophila* comb. nov. *FEMS Yeast Research*, 5(4–5), 463–469.

Visvanathan, R., Qader, M., Jayathilake, C., Jayawardana, B. C., Liyanage, R., & Sivakanesan, R. (2020). Critical review on conventional spectroscopic α-amylase activity detection methods: merits, demerits, and future prospects. *Journal of the Science of Food and Agriculture*, 100(7), 2836–2847.

Wickerham, L. J. (1951). Taxonomy of yeasts (No. 1029). US Department of Agriculture.

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, 18(1), 315–322.

Zabed, H., Sahu, J. N., Suely, A., Boyce, A. N., & Faruq, G. (2017). Bioethanol production from renewable sources: Current perspectives and technological progress. *Renewable and Sustainable Energy Reviews*, 71, 475–501.

Zapata-Castillo, P., Villalonga-Santana, L., Islas-Flores, I., Rivera-Muñoz, G., Ancona-Escalante, W., & Solís-Pereira, S. (2015). Synergistic action of laccases from *Trametes hirsuta* Bm2 improves decolourization of indigo carmine. *Letters in Applied Microbiology*, 61(3), 252–258.

Zhang, J., Ke, W., & Chen, H. (2020). Enhancing laccase production by white-rot fungus *Trametes hirsuta* SSM-3 in co-culture with yeast *sporidiobolus pararoseus* SSM-8. *Preparative Biochemistry & Biotechnology*, 50(1), 10–17.