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Research Article

Industrial Yeast Characterisation for Single Cell Protein Application

Maria Clara De-Barros, Júlia Bizerra-Santos, Luanna Maia, Normando Ribeiro-Filho*

Laboratory of Agro-industrial Products Technology, Department of Soil and Rural, Centre for Agrarian Science, Campus II, Federal University of Paraiba, Areia, Paraiba, Brazil
Email: normandofilho@cca.ufpb.br

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Abstract: Food waste and protein for food and feed are among the worldwide concerns. For years, yeast cells have been used for food production; and lately, have been applied for protein enrichment and for food recovery. Breweries, wineries, and distilleries generate a large amount of yeasts that are mostly wasted. Moreover, food loss that includes fruit waste is also a world concern, therefore, identifying the characteristics of yeast cells to drive its application to the correct source can be useful for reducing fruit waste and yeast waste. Therefore, this work aimed to evaluate the potential of yeast strains used by breweries, wineries, and distilleries for future application as single cell proteins including food waste recovery, sustainable food processing, protein for vegetarian products, or world's protein necessities. Two *Saccharomyces cerevisiae* (NCYC2592 and M2), and one *Saccharomyces pastorianus* (W34/70) were evaluated by using traditional and modern methods for evaluating four tests including permissive growth temperature, capacity to grow on different carbon sources, DNA fingerprint (physiological differentiation), and mineral content. NCYC2592 can grow at 40 °C. W34/70 can grow on melibiose and rhamnose; NCYC2592 and M2 could not grow on these carbohydrates. P, K and Mg are the most abundant minerals present in all strains. Yeasts that contain higher concentration of macro-minerals such as P, K, and Mg can grow under higher temperatures.

***Keywords*:** protein concern, food waste, brewing yeast, future application

1. Introduction

Proteins are the basic building blocks required for living cells' growth and development [1]. They are responsible for forming nucleic acids and enzymes, and carry out a variety of biochemical reactions, which occur in vegetables, cereals, and fruits [2-3]. Because of population growth and market demands (primarily vegetarian food), the global demand for food and feed proteins continues to rise, therefore, alternative protein sources are in need [2]. Food waste (food loss) is also a global concern as a result of an agricultural process (including technical limitations in storage, infrastructure, packaging, and marketing) [2-4]. The concept of sustainability is built on three pillars: economic, environmental, and social [4]. As a result, sustainable food production is critical to maintaining development and eradicating hunger. As an alternative, scientists have been working on new techniques for recovering protein from food waste including tropical fruits [2].

The processing of tropical fruits generates a significant amount of food waste that is well known. Fruit waste contains high concentration of carbohydrates, fiber; asks, moisture, water activity, fibers, hemicellulose, pectin, amino

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acids, and protein [2]. All these compositions can be used as a great environment for microbial protein production. Humans have been using single cells for food production (including alcoholic beverages, bread, cheese, yogurt, and soy sauce), which raised the idea of using single-cell for protein enrichment and food recovery via semi-solid-state fermentation (SSSF) [2, 5]. The increasing world demand for protein rich food led to the search for alternative protein sources to supplement the conventional protein sources, therefore, single cell protein (SCP) became an innovative alternative to help the global protein concern [6-7]. Microorganisms such as algae and yeasts started to be used as microbial proteins due to its easy to manipulate [5]. For increasing the protein content in food waste, bioprocesses such as SSSF have been successfully used as a technique for food waste recovery.

Yeasts (*Saccharomyces* genus) are commonly utilised in breweries, wineries, and distilleries. Understanding yeast behavior through the use of various carbon sources and temperature during yeast growth is critical for process control [8-9]. Brewing yeasts can metabolise most carbohydrates including glucose, fructose, sucrose, maltose, galactose, mannose, raffinose, and trehalose, but they cannot digest dextrin [9-11]. Yeast strains used for the production of beer type ale and beer type lager can be distinguished by their ability to consume melibiose (disaccharide) [12]. Furthermore, temperature is an important parameter for process control because indicates the best conditions for yeasts survival as well as process limitations. Permissive growth temperature is supported by the fact that *Saccharomyces* can or cannot grow at 37 °C [12-13]. *S. cerevisiae* can grow at 37 °C, but other brewing-associated species such as *S. pastorianus* cannot [13]. Currently, a large quantity of brewing yeasts are generated from breweries, wineries, and distilleries, which are mostly treated as waste. Although several works has been conducted on single cell protein technology. From the best of our knowledge, breweries, wineries, and distilleries generate a large amount of yeasts, which most of it is wasted. Therefore, this work aimed to evaluate the potential of yeast strains used by breweries, wineries, and distilleries for future application as single cell proteins including food waste recovery, sustainable food processing, protein for vegetarian products, or world's protein necessities.

2. Material and method

2.1 *Media, and solutions*

All media and solutions were prepared using materials supplied by Fisher Scientific, UK. All media were sterilised at 121 °C at 15 psi for 15 min immediately after preparation.

Yeast Peptone Dextrose (YPD): YPD (1% of yeast extract, 2% of peptone and 2% of D-glucose) was used in the most of experiments for yeast growth. YPD was adjusted up to 1litre using RO (reverse osmosis) water, sterilised (steam, 121 °C) and stored at 22 °C (room temperature).

Yeast Peptone (YP) + SCS (specific carbon source): YP + SCS (specific carbon source) was prepared at a concentration of 2% (specific carbon source). The carbon sources used were glucose, fructose, galactose, xylose, mannose, rhamnose, sucrose, maltose, lactose, melibiose and melezitose. All media were adjusted up to 1 litre using RO (reverse osmosis) water and sterilised (steam, 121 °C) and stored at 22 °C (room temperature).

YPD agar: Dissolving 1% of yeast extract, 2% of peptone, 2% of D-Glucose and 2% agar adjusted up to 1litre using RO (reverse osmosis) water.

Methylene blue viability assessment: 100 mL of methylene blue (0.01 g/L stock solution) was dissolved in sodium citrate solution (2% w/v) to a final concentration of 0.01% [14].

2.2 *Microorganism assays*

Yeast Strains and Storage: Two yeast *Saccharomyces cerevisiae* strains (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70) were used in these study. All strains used in this study were fresh yeast, which were laboratory-grown at 25 °C at 150 RPM for 24 hours. Stock cultures of each strain were prepared and maintained in: 1) Cryovials (Nalgene Nunc International, UK) stored at -80 °C. A loop full of yeast grown on YPD agar was re-suspended in cryoprotectant solution containing 1 ml YPD plus 20% (v/v) glycerol (Sigma, UK) to maintain cell viability; and 2) using a small aliquot of the stock cryopreserved culture, which were propagated in 10 mL and 250 mL of YPD at 25 °C for 3 days each. Therefore, YPD agar slopes were prepared and maintained as stock culture at 4 °C during 2 months for use in subsequent experiments.

Microorganism propagation: The fermentation used three *Saccharomyces* yeast strains were used in the fermentation, two strains belonged to the *Saccharomyces cerevisiae* species (NCYC2592 and M2) and one *Saccharomyces pastorianus* (W34/70). Microorganism were inoculated in 2%YPD and incubated for 24 hours at 30 °C.

Viability assays: Yeast suspension (0.5 ml) was mixed with methylene blue solution (0.5 ml) and after 5 min at room temperature examined microscopically (Leitz, Diaplan Microscope, Germany at x400 magnification). Images were captured using a digital camera attached to the microscope. Viability was measured using Aber CountStar instruments connected to a computer for data acquisition by CountStar software. The CountStar instrument is used with specific disposable slides containing 5-sample chambers and require 20 µl for each sample (Aber CountStar Instruments specifications) [15].

2.2.1 *Measurement of yeast growth*

Yeasts growth measurement were performed in 96 well plates covered with low evaporation film (Costa®) and monitored using a Tecan (Mannedorf, Switzerland) Infinite M200 Pro plate reader (OD600 nm). Tecan measurements were divided into four growth assays: 1) Yeast strains differentiation based on growth temperature, and 2) yeast growth and differentiation on different carbon source. All fermentations were conducted in triplicate.

2.2.1.1 *Growth temperature assay*

Ale and lager strain differentiation based on growth temperature. Yeast growth were measured in 96-well plates at 22 °C, 25 °C, 30 °C, 37 °C and 40 °C for 72 h. Yeasts were inoculated 1.5×10^7 cells/mL. All growths were classified and described into Table 1.

Table 1. Growth intensity classification using Tecan growth curves.

OD600	Growth intensity	Representation
0.8-1	very intense growth	++++
0.6-0.79	intense growth	+++
0.4-0.59	moderate growth	++
0.3-0.39	low growth	+
0-0.29	no growth	-

2.2.1.2 *Yeast growth on different carbon sources*

Ale and lager strain differentiation based on carbon source utilization**.** Yeast growth was measured in 96-well plates. Individual strains were inoculated on different carbon source for 72 h at 25 °C. YP (2% bacteriological peptone, 1% yeast extract) + 2% of individual carbon source (glucose, fructose, galactose, xylose, mannose, rhamnose, sucrose, maltose, lactose, melibiose, melezitose). Yeasts were inoculated 1.5×10^7 cells/mL. All growths were classified and described into Table 1.

2.3 *DNA assay*

2.3.1 *DNA extraction*

DNA was extracted from 10 ml YPD incubated at 25 °C at 150 RPM for 24 h. 2 ml of the culture was centrifuged in a 2.5 ml Eppendorf tube (5,000 rpm, 5 min). 400 µl of lysis buffer (Tris 10 mM, pH 7.6, EDTA 1 mM, NaCl 100 mM, Triton X-100, 2% w/v, sodium dodecyl sulphate (SDS) 1% w/v), 400 µl of phenol/chloroform/iso-amyl alcohol (25:24:1

v/v), and 600 µl of glass beads were added to the pellet. The mixture was vortexed for 4 min. Then 200 µl of Tris EDTA (pH 7.6) buffer was added, and the mixture centrifuged for 5 min at 6,000 rpm. 500 µl of chloroform:isoamyl alcohol (98:2 v/v) was added to the upper phase and after gentle agitation, the mixture was submitted to centrifugation (14,000 rpm for 2 min). Two volumes of ethanol were added to the aqueous phase. After centrifugation (14,000 rpm, 5 min), the nucleic acid pellet was dissolved in 10 mM TE buffer pH 8.0 [16].

2.3.2 *DNA amplification*

Polymerase chain reaction (PCR) amplifications were carried out in 25 µl reaction volumes containing 5-20 ng yeast DNA, 10 mM Tris pH, 9.0, 50 mM KCl, 0.1% Triton X-100, 0.2 mg/ml gelatin, 200 mM of each deoxynucleoside triphosphate (dNTP), 2.5 mM $MgCl_2$ and 1 µM for each oligonucleotide primer of the delta12 family and delta21 family. Primers are delta12 (5'-TCAACAATGGAATCCCAAC-3') and delta21 (5'-CATCTTAACACCGTATATGA-3'). Amplification reactions were performed with a Stratagene thermal cycler using the following programme: 4 min at 98 °C followed by 38 cycles of 10 s at 98 °C, 30 s at 50 °C and 90 s at 72 °C and the finishing step of 10 min at 72 °C [16].

2.3.3 *Electrophoresis*

Amplification products were separated by electrophoresis on 15 cm of 2% agarose gels submitted to 75 mV for 1.5 h in 1xTBE buffer and fluorescent ethidium bromide. The stained bands were photographed under UV light using VisionWorks®LS analysis and edition was treated using adobe Photoshop.

2.4 *Inductively Coupled Plasma Mass Spectrometry (ICP-MS) protocol*

2.4.1 *Sample digestion*

50 ml of fermented samples were centrifuged, and the supernatant was separated. A total of 0.25 g dried yeast (solid sample) was taken and 5 mL of HNO_3 was added. All samples were kept at room temperature for 24 hours. Microwave heat was applied to the samples (Multiwave PRO, Anton Paar) using the following temperature program: power (1,500 W), heating to 140 °C in 10 minutes, holding the temperature at 140 °C for 30 minutes, and cooling for 30 minutes. The digested samples were transferred to new tubes and adjusted to a volume of 20 ml with milliQ water. 1 ml of diluted samples were transferred into ICP-MS tubes and adjusted to a volume of 10 ml with milliQ water. All samples preparation for ICP-MS analysis followed the recommendation from Ribeiro-Filho et al. [17].

2.4.2 *ICP-MS analysis*

The following parameters were used with the ICP-MS instrument: The plasma power is 1,550 W; the cool gas flow rate is 14 L/min; the argon gas flow rates are auxiliary at 0.8 L/min and nebulizer at 0.4 L/min; and the sample flow rate is 4.0 mL/min. ICP-MS was used to perform multi-element analysis on diluted solutions (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific, Bremen, Germany). The instrument was operated in three modes: I a collision-cell (Q cell) using He with kinetic energy discrimination (He-cell) to remove polyatomic interferences, (ii) standard mode (STD) in which the collision cell is evacuated, and (iii) hydrogen mode (H_2-cell) using H_2 gas as the cell gas. Samples were introduced via a PEEK nebulizer from an autosampler (Cetac ASX-520) equipped with an ASXpress^{TM} rapid uptake module (Burgener Mira Mist). Internal standards, Ge (10 g L^{-1}), Rh (10 g L^{-1}) and Ir (5 g L^{-1}) in 2% trace analysis grade (Fisher Scientific, UK) HNO3, were introduced to the sample stream on a separate line via the ASXpress unit. External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) in the range 0-100 g L^{-1} (0, 20, 40, 100 g L^{-1}) included Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Cu, Fe, K, Li, Mg, Mn, Mo, Na, Ni, P, Pb, Rb, and S. Ca, Mg, Na, and K standards in the range 0-30 mg/L were created using a custom external multi-element calibration solution (PlasmaCAL, SCP Science, France). In-house standard solutions were used for phosphorus, boron, and sulphur calibration (KH_2PO_4, K_2SO_4 and H_3BO_3). B and P were measured in STD mode, Se in H2-cell mode, and all other elements in He-cell mode using in-sample switching. QtegraTM software (Thermo-Fisher Scientific) was used to process the samples, with external cross-calibration between pulse-counting and analogue detector modes used when necessary. All ICP-MS analysis followed the recommendation from Ribeiro-Filho et al. [17]. All mineral composition analysis were conducted in triplicate.

2.5 *Statistical analysis*

Results were analysed by Analysis of variance (ANOVA) with Tukey's post hoc test to identify significant differences ($p < 0.05$) using XLSTAT® and Microsoft excel®. Principal Component Analysis (PCA) was used to reduce the scope of dimension of the complex multivariate data sets for ease interpretation and identifying trends/correlations among treatments and samples. PCA was conducted for 5 temperatures and 35 minerals and was carried out using XLSTAT and Microsoft excel®. Kaiser Criteria (eigenvalue > 1) and cumulative variance (> 60%) was used to describe/select data, which is summarised in the PCA Bi-plot [15, 17].

3. Results

In this study, two ale yeast strains, NCYC2592 (*Saccharomyces cerevisiae*) and M2, and one lager strain, W34/70 (*Saccharomyces pastorianus*), were used. Traditional and modern methods were used to confirm the lager and ale phenotypes of NCYC2592, W34/70, and M2. Yeast strains were evaluated using four different tests: permissive growth temperature, capacity to grow on different carbon sources, DNA fingerprint (physiological differentiation), and mineral content.

3.1 *Permissive growth temperature*

Traditional *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* differentiation is measured using a temperature assay, which assumes that *Saccharomyces pastorianus* strains cannot grow at 37 °C but *Saccharomyces cerevisiae* can grow above 37 °C (18, 9). Our method provides five growth levels: OD 0.8-1 (++++ very intense growth); OD 0.6-0.79 (+++ intense growth); OD 0.4-0.59 (++ moderate growth); OD 0.3-0.39 (+ low growth); and OD 0-0.3 (- no growth). Results demonstrated that W34/70 and M2 grow similarly at 22 °C, while NCYC2592 grows significantly better than the other two strains (Table 2). All strains grow similarly at 25 °C. At 30 °C, NCYC2592 (0.93 OD) grows faster than M2 (0.89 OD), and W34/70 (0.85 OD) grows similarly to both other strains (Table 2). NCYC2592 and M2 can grow at 37 °C, but W34/70 cannot (0.20 OD). NCYC2592 can grow at 40 °C, but M2 cannot (Table 2).

Table 2. Yeast ability to grow in different temperature for 72 h.

Temp.	OD600			Growth intensity		
	NCYC2592	W34/70	M2	NCYC2592	W34/70	M2
22 °C	0.92^{a}	0.88^{b}	0.84^{b}	++++	++++	++++
25 °C	0.99^{a}	0.95^{a}	0.97^{a}	++++	++++	++++
30 °C	0.93^{a}	0.85^{ab}	0.89^{b}	++++	++++	++++
37 °C	0.95^{a}	0.20^{c}	0.84^{b}	++++	-	++++
40 °C	0.89^{a}	0.00^{b}	0.01^{b}	++++	-	-

Data from triplicate measurements presented in average. Data with different letters are significantly different at $p < 0.05$.
++++ very intense growth (OD 0.80-1.00)
+++ intense growth (OD 0.60-0.79)
++ moderate growth (OD 0.40-0.59)
+ low growth (OD 0.3-0.39)
- no growth (OD 0-0.29)
*Any growth under 0.39 OD can be ignored because it cannot be visualised on the spot plate.

3.2 *Yeast growth on different carbon sources*

To assess yeast growth on various carbon sources, YP (1% yeast extract and 2% peptone) + SCS (2% specific carbon source) were used. Glucose, fructose, galactose, xylose, mannose, rhamnose, sucrose, maltose, lactose, melibiose, and melezitose were the carbon sources utilised (Table 3). The growth curves were created using OD600 at 25 °C, which was determined to be the optimal growth temperature for all strains in this study. Our method consider five growth levels were considered: OD 0.8-1 (++++ very intense growth); OD 0.6-0.79 (+++ intense growth); OD 0.4-0.59 (++ moderate growth); OD 0.3-0.39 (+ low growth); and OD 0-0.3 (- no growth) (Table 3). When inoculated in medium containing glucose or fructose as a carbon source, all strains grew rapidly (OD 0.8-1.0) (Table 2). When galactose, mannose, sucrose, or maltose were used as carbon sources, all strains grew as a very intense (OD 0.8-1), except W34/70 grew as an intense growth (OD 0.60-0.79) (Table 3). W34/70 is capable of growing on melibiose (0.81 OD) or rhamnose (0.72 OD); in contrast, other strains could no grow on melibiose or rhamnose (Table 3).

Table 3. Yeast growing using different carbon sources with a concentration of 2% at 22 °C during 72 h

Carbon source	OD600			Growth intensity		
	NCYC2592	W34/70	M2	NCYC2592	W34/70	M2
Glucose	0.89^{a}	0.81^{b}	0.86^{ab}	++++	++++	++++
Fructose	0.92^{a}	0.81^{b}	0.89^{a}	++++	++++	++++
Galactose	0.84^{a}	0.77^{b}	0.83^{a}	++++	+++	++++
Xylose	0.11^{a}	0.10^{a}	0.09^{a}	-	-	-
Mannose	0.85^{a}	0.73^{b}	0.83^{a}	++++	+++	++++
Rhamnose	0.15^{b}	0.72^{a}	0.13^{b}	-	+++	-
Sucrose	0.87^{a}	0.78^{b}	0.85^{a}	++++	+++	++++
Maltose	0.80^{a}	0.74^{b}	0.78^{ab}	+++	+++	+++
Lactose	0.55^{c}	0.60^{bc}	0.75^{a}	++	+++	+++
Melibiose	0.38^{b}	0.81^{a}	0.23^{c}	++	++++	-
melezitose	0.51^{a}	0.58^{a}	0.49^{a}	++	++	++

Data from triplicate measurements presented in average. Data with different letters are significantly different at $p < 0.05$.
++++ very intense growth (OD 0.80-1.00)
+++ intense growth (OD 0.60-0.79)
++ moderate growth (OD 0.40-0.59)
+ very low growth (OD 0.3-0.39)
- no growth (OD 0-0.29)
*Any growth under 0.39 OD can be ignored because it cannot be visualised on the spot plate.

All strains cannot grow on xylose (OD 0-0.3) and showed a moderate growth (OD 0.40-0.60) when inoculated with melezitose (Table 3). Using lactose as a carbon source, NCYC2592 (0.55 OD) showed a moderate growth; however, W34/70 (0.60 OD) and M2 (0.75 OD) showed an intense growth (OD 0.6-0.8) (Table 3). Except for rhamnose and melibiose, W34/70 (*S. pastorianus*) grows significantly less than the other two *S. cerevisiae* strains (NCYC2592 and

M2, ale strains) on all carbohydrates used in this study. As a single carbon source, W34/70 can grow rhamnose and melibiose. As a result, can support the distinction and confirms that NCYC2592 and M2 are ale strains (*Saccharomyces cerevisiae*) and W34/70 is a lager strain (*Saccharomyces pastorianus*) (Table 3).

3.3 *Differentiation of yeast strains*

Our data stated that yeasts used by breweries (W34/70), wineries (M2), and distilleries (NCYC2592) behaved differently during their growth on different temperature, and different carbon sources. In this section, DNA *fingerprint* is provided because identifies that yeast strains evaluated contain an unique DNA and confirms that they are genetically different. Therefore, DNA fingerprint helps to explain the different behaviour of yeast strains during its growth on different temperature, and different carbon sources. DNA fingerprinting is a technique used in genetic engineering that aids in the verification of DNA compatibility. In theory, DNA is a molecule that accumulates in the nucleus of a cell, all genetic information that varies from individual to individual. The standard of bands is unique to each individual, and similarities demonstrate genetic proximity. According to Legras and Karst [16], strains *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* can be identified using delta12-delta21 primers for DNA *fingerprinting*. The results are shown in electrophoresis patterns obtained using agarose electrophoresis with different yeasts and delta12-delta21 primers, which demonstrate the detection of intense and faint bands (Figure 1). NCYC2592 and M2 have intense and similar bands from 140 bp to 400 bp, which was expected given their ability to grow at 37 °C (Table 2) and lack of ability to grow on melibiose (Table 3). In contrast, The DNA fingerprint of M2 displayed five intense bands over 400bp that differ from NCYC2592 and W34/70. W34/70, on the other hand, has other faint bands below 140 bp and above 500 bp, which is completely different from the other two strains used in this study. These three yeast strains each had a unique DNA fingerprint, confirming that they are genetically distinct.

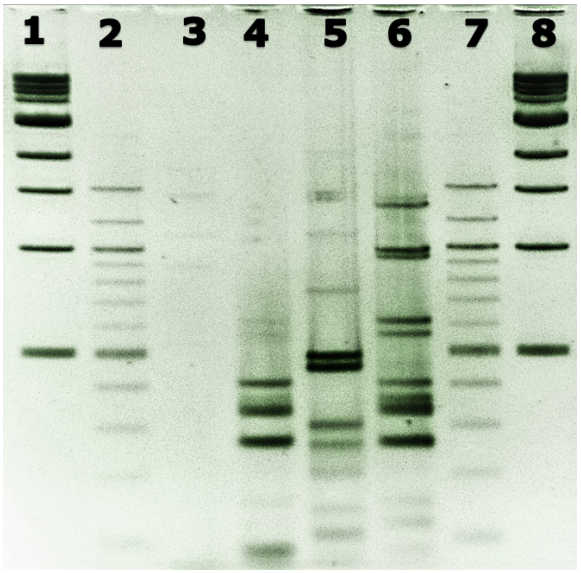

Figure 1. Electrophoretical patterns carried for different yeasts with delta12-delta21 primers. Lanes 1-8 of gel: 1, molecular mass marker (1 Kb ladder); 2, molecular mass marker (100 pb ladder); 3, negative control; 4, *S. cerevisiae* NCYC2592; 5, *S. pastotianus* W34/70; 6, *S. cerevisiae* M2; 7, molecular mass marker (100 pb ladder); 8, molecular mass marker (1 Kb ladder). Pictures' capture in dark incubator under UV light using VisionWorks®LS Analysis Software editing using adobe Photoshop.

3.4 *Yeast mineral content*

In our previous work, we investigated the effect of essential inorganic element supplementation (including nitrogen, phosphorus, potassium, magnesium, copper, zinc, iron, and manganese) on flavour generation (including ethanol, glycerol, higher alcohols, and esters) during yeast fermentation [17]. The authors observed that when the

wort was supplemented with ammonia-nitrogen, inorganic phosphate, potassium, or magnesium, yeasts produced more flavour compounds without affecting their growth/cell viability. In contrast, when the wort was supplemented with heavy metal ions a negative effect on flavour formation, and yeast growth/cell viability were observed. Growth temperature influences on carbon source consumption is well-known. For growing, yeasts require carbon source, amino acids, and minerals (as co-factors) as the vital sources; therefore, yeast mineral composition can indicate the application of the yeast strain, and can help to explain the different behaviour of yeast strains during its growth on different temperature, and different carbon sources. The mineral composition of yeast was classified into three groups: Group 1 contains nine nutrients that are essential for yeast health and growth. Group 2 (presented in ppm-mg/Kg) contains a very important group of elements that are responsible for metabolic activities and help driving elements from group 1 into yeast cells, but if intakes of group 2 elements do not occur, yeast growth may not be affected (Table 4). Finally, group 3 (represented in parts per billion-µg/Kg) includes mineral elements, which are present in very low concentrations in yeast and wort. W34/70 contains a lower significant amount of phosphorus (16.90 g/kg) than M2 (20.7 g/kg), but a similar amount when compared to NCYC2592 (19.40 g/kg) (Table 4). NCYC2592 contains a higher potassium concentration (27.3 g/kg) than W34/70 (18.1 g/kg) and M2 (17.3 g/kg) (Table 4). M2 has a significantly higher calcium concentration (635 mg/kg) than NCYC2592 (290 mg/kg) and W34/70 (349 mg/kg) (Table 4). All strains evaluated contain a different concentration of copper. M2 (5.9 mg kg) contains a higher copper content than W34/70 (4.4 mg/kg) and NCYC2592 (1.86 mg/kg). NCYC2592 comprises a lower zinc content (304 mg/kg) than W34/70 (508 mg/kg) and M2 (470 mg/kg) (Table 4). All strains contain the same amount of magnesium, iron, and manganese (Table 4).

Table 4. Mineral composition of NCYC2592, W34/70 and M2 strains.

Minerals	Unit.	Yeasts stain		
Group 1		NCYC 2592	W34/70	M2
Nitrogen*	mg/kg	-	-	-
Phosphorus		$19,405^{ab} \pm 437$	$16,912^{b} \pm 506$	$22,656^{a} \pm 2,007$
Potassium		$27,366^{a} \pm 472$	$18,064^{b} \pm 243$	$17,281^{b} \pm 1,435$
Magnesium		$1,109^{a} \pm 20$	$1,056^{a} \pm 12$	$1,063^{a} \pm 85$
Calcium		$290^{b} \pm 3$	$349^{b} \pm 7$	$635^{a} \pm 50$
Iron		$159^{a} \pm 3$	$164^{a} \pm 2$	$144^{a} \pm 13$
Copper		$1.86^{c} \pm 0.04$	$4.4^{b} \pm 0.1$	$5.9^{a} \pm 0.5$
Zinc		$304^{b} \pm 17$	$508^{a} \pm 7$	$470^{a} \pm 4$
Manganese		$249^{a} \pm 184$	$603^{a} \pm 137$	$245^{a} \pm 85$
Group 2				
Sulphur	mg/kg	$4,565^{a} \pm 118$	$4,777^{a} \pm 80$	$3,528^{b} \pm 277$
Sodium		$3,492^{b} \pm 88$	$15,742^{a} \pm 379$	$13,604^{a} \pm 1,232$
Aluminium		$0.7^{a} \pm 0.1$	$0.4^{a} \pm 0.2$	$0.3^{a} \pm 0.0$
Boron		$0.74^{a} \pm 0.03$	$0.53^{b} \pm 0.01$	$0.51^{b} \pm 0.07$
Rubidium		$7.2^{a} \pm 0.2$	$6.68^{a} \pm 0.06$	$7.8^{a} \pm 0.7$
Strontium		$1.84^{b} \pm 0.05$	$2.14^{b} \pm 0.03$	$2.85^{a} \pm 0.25$

Table 4. (cont.)

Minerals	Unit.	Yeasts stain		
Group 3		NCYC 2592	W34/70	M2
Barium	µg/kg	$323^{a} \pm 16$	$215^{c} \pm 8$	$267^{b} \pm 22$
Titanium		$118^{b} \pm 5$	$137^{b} \pm 11$	$199^{a} \pm 16$
Chromium		$15^{a} \pm 2$	$25^{a} \pm 2$	$23^{a} \pm 21$
Cobalt		$2,987^{b} \pm 56$	$6,356^{a} \pm 67$	$3,102^{b} \pm 248$
Nickel		$51^{a} \pm 7$	$49^{a} \pm 6$	$28^{a} \pm 11$
Lithium		$91^{c} \pm 2$	$157^{a} \pm 6$	$127^{b} \pm 13$
Beryllium		$0.7^{a} \pm 0.6$	$0.4^{a} \pm 0.1$	$0.7^{a} \pm 0.4$
Vanadium		$153^{a} \pm 5$	$116^{b} \pm 8$	$102^{b} \pm 7$
Gallium		$10^{a} \pm 7$	$20^{a} \pm 4$	$6.8^{a} \pm 2.4$
Arsenic		$107.4^{a} \pm 3.6$	$60.2^{b} \pm 5.4$	$54.2^{b} \pm 4.7$
Selenium		$140.9^{a} \pm 6.5$	$132.7^{a} \pm 0.6$	$125^{a} \pm 9$
Molybdenum		$32.8^{a} \pm 0.9$	$34.5^{a} \pm 0.7$	$33.4^{a} \pm 5.1$
Silver		$1.4^{a} \pm 0.6$	$1.8^{a} \pm 0.1$	$1.7^{a} \pm 0.6$
Cadmium		$28.3^{c} \pm 2.2$	$59^{a} \pm 2$	$41^{b} \pm 2$
Caesium		$57^{c} \pm 1$	$72^{b} \pm 1$	$92^{a} \pm 6$
Thallium		$74^{a} \pm 2$	$25.7^{b} \pm 0.5$	$24^{b} \pm 2$
Lead		$6^{a} \pm 1$	$0.8^{a} \pm 0.6$	$3.0^{a} \pm 2.8$
Uranium		$1.9^{b} \pm 0.1$	$3.00^{a} \pm 0.03$	$2.0^{b} \pm 0.2$

*Ammonium nitrogen was not analysed
**Data highlighted in orange colour represent the similarities among three yeast strains evaluated.
Data from triplicate measurements presented in average and standard deviation. Data with different letters are significantly different at $p < 0.05$.

3.5 *Overall, of yeast differentiation*

When comparing permissive growth temperature and mineral content, principal component analysis (PCA) reveals that all yeasts used in this study are very different. There are correlations between permissive growth temperature and mineral content. Figure 2 shows how the first component distinguishes traditional lager (W34/70) strains from ale strains (M2 and NCYC2592). The second component distinguishes ale (M2) from lager (W34/70) and ale (NCYC2592). Considering the temperature of 37 °C used for differentiation via permissive growth, PCA separates S*accharomyces cerevisiae* strains from *Saccharomyces pastorianus* and, as expected, illustrates that *S. cerevisiae* (ale strains) grew better than *S. pastorianus* (lager strains) at all temperatures evaluated.

The separation is explained by the first two components (PCA1 and PCA2). D1 and D2 represent PCA1 and PCA2, respectively. Figure 2 depicts the global analysis components as specific correlations to each yeast strain studied. As a result, all yeasts evaluated were physiologically distinct in terms of permissive growth temperature, carbon source, and mineral composition, as confirmed by DNA fingerprinting. W34/70 contains a high concentration of ions such

as sodium, zinc, manganese, and aluminium, but a low concentration of ions such as potassium, magnesium, and barium, as illustrated by the PCA (Figure 2). Yeasts like NCYC2592 and M2 that grow at 37 °C and 40 °C have higher concentrations of potassium, magnesium, and phosphorus, but lower concentrations of heavy metals such as iron, lead, nickel, and selenium.

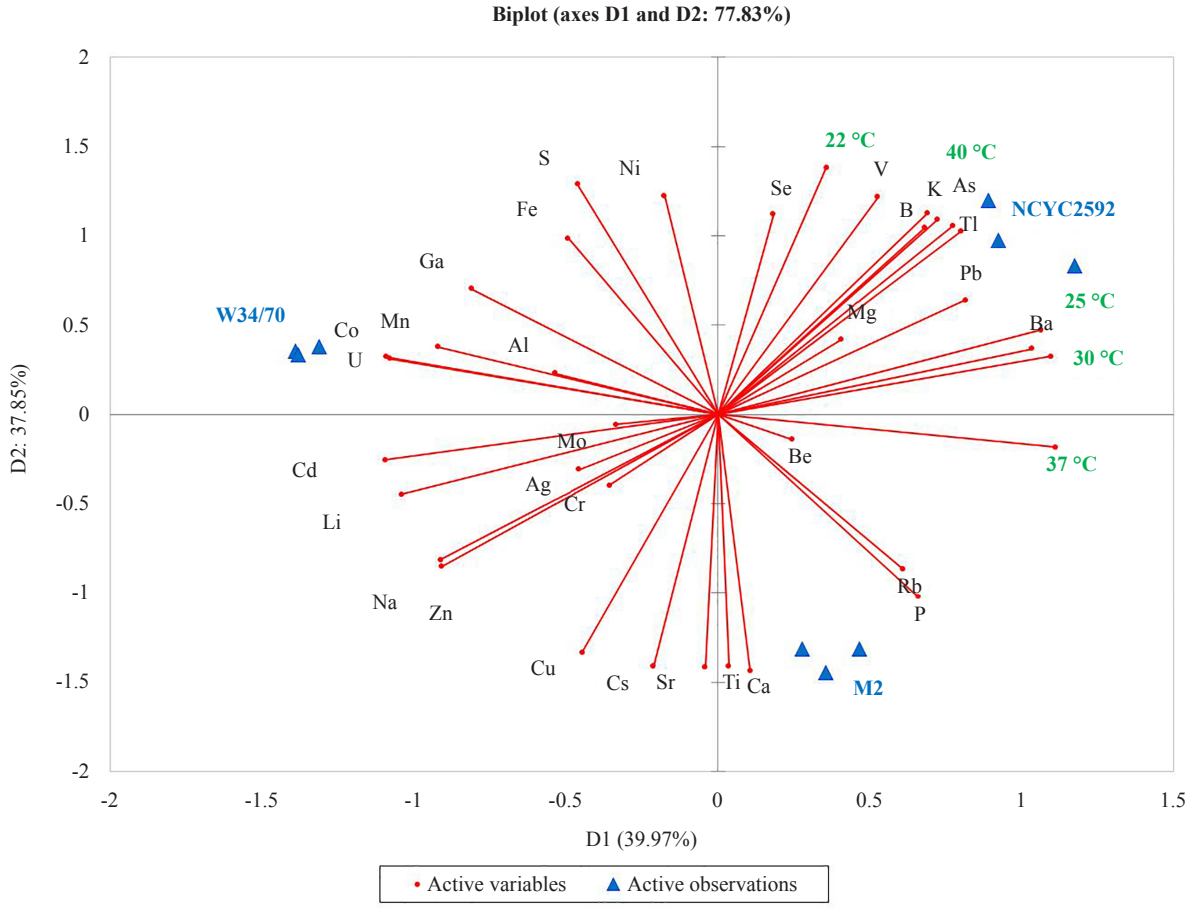

Figure 2. Principle component analysis (Bio-Plot) to identify the correlation and similarities among all 37 variables including 32-mineral elements, 5-growing temperature and 3 yeast strains (NCYC2592, W34/70 and M2).

4. Discussion

Brewing yeast differentiation is important because it supports physiologic and genetic differences. As a result, it aids in processing control and, as a result, product quality. After comparing more than 40 brewing yeast *Saccharomyces* strains (ale and lager), Kopecká et al. [18] concluded that *Saccharomyces pastorianus* cannot grow at 37 °C and *Saccharomyces cerevisiae* can [8-9, 18-19]. All of the yeasts used in this study can grow at temperatures ranging from 15 to 30 degrees Celsius. W34/70 strain can grow at temperatures ranging from 15 °C to 35 °C, but not at 37 °C, classifying it as *Saccharomyces pastorianus* (lager strain) (Table 2). In contrast, NCYC2592 and M2 are ale strains capable of growing at 37 °C (Table 2). However, only NCYC2592 can grow at 40 °C, which explains why this strain has been used for bioethanol production due to its ability to grow at temperatures ranging from 40 to 45 °C [20].

The ability of yeast to consume hexoses via glycolysis and convert them to ethanol is well known. Yeast grows on a variety of carbon sources including monosaccharides and disaccharides [9, 20]. lager yeast strains and ale yeast strains can both grow on hexoses (monosaccharides) because its cell wall can transport disaccharides and is broken

down in hexoses. All of these carbohydrates are broken down by glycolysis [17]. *Saccharomyces* strains have an advanced hexose transport system that regulates carbohydrate transport and has a high affinity for glucose [11, 20-21]. *S. cerevisiae* ferments glucose, mannose, and fructose via glycolysis, and galactose via a combination of the Leloir pathway and glycolysis [21].

Although glucose is the yeast-*Saccharomyces* preferable carbon/energy source [20]. Furthermore, fructose and mannose are isomers of glucose that all yeast strains can grow on and are transported by the same hexose transporters [20-21]. All three yeast strains used in this study grew on glucose and its isomers (mannose and fructose), as expected. Moreover, *Saccharomyces cerevisiae* grows on galactose, which is first absorbed by the galactose permease (Gal2p), and then converted to glucose-6-phosphate [9, 20]. All three yeast strains used in this study can grow on galactoses; therefore, they consume galactose via a combination of the Leloir pathway and glycolysis [21].

Glucose and fructose are both referred to as "rapid fermentable sugars" or "reduced sugars," and rhamnose is more reduced than either [11, 21]. However, due to the lack of genes encoding rhamnose-metabolizing enzymes in its genome; therefore, yeast *S. cerevisiae* cannot grow on rhamnose as the sole carbon source [9, 21]. As a result, NCYC2592 and M2 strains, as expected, cannot grow on rhamnose as a carbon source (Table 2), supporting their classification as *Saccharomyces cerevisiae* strains. W34/70, on the other hand, can grow on rhamnose (Table 3), suggesting that this strain contains rhamnose-metabolizing enzymes.

Saccharomyces strains can ferment disaccharides maltose (genes encoded MAL1-4 and MAL6) and sucrose (invertase genes encoded SUC1-5 and SUC7), but they can use lactose and melezitose as a carbon source as well [8-9, 11]. As expected, all strains can grow on maltose, sucrose, lactose or melezitose as single carbon source (Table 3). However, when inoculated on lactose or melezitose as a single carbon source, all strains showed a significant reduction in their growth when compared to maltose and sucrose as single carbon source. According to van Maris et al. [21] yeast growth on lactose and melezitose as a single substrate is inconsistent, therefore, our results classified as a moderate growth (OD 0.4 0.6) (Table 3).

The majority of carbohydrates can be used by brewery yeast; however, ale (*Saccharomyces cerevisiae*) and lager (*Saccharomyces pastorianus*) can be distinguished by their ability to assimilate or not assimilate melibiose (disaccharide) [11, 20]. As a result, ale strains cannot grow on melibiose, whereas lager strains can. These occurred due to the presence of -D-galactose activity in lager strains, which hydrolyses melibiose into galactose and glucose [8, 11]. M2 did not grow on melibiose as a single carbon source because they lack -D-galactose activity and thus cannot convert melibiose into galactose and glucose [9, 11]. NCYC2592 reveals a very low growth (O.D. 0.38) on melibiose as a single carbon source; however, this growth is low comparing to the very intense growth, which *Saccharomyces pastorianus* exhibits. In contrast, W34/70 (OD 0.81) exhibits very intense growth (OD 0.8-1) (Table 4) on melibiose due to the presence of -D-galactose activity, which converts melibiose to galactose and glucose (10, 8). W34/70 (*S. pastorianus*) grows significantly less than the other two *S. cerevisiae* (NCYC2592 and M2, ale strains) on all carbohydrates used in this study, except on rhamnose and melibiose, which W34/70 showed an intense growth on rhamnose (OD 0.72) and a very intense on melibiose (OD 0.81) was used as a single carbon source (Table 3). As a result, the different growth behavior of all strains inoculated on different carbon sources supports thermal differentiation and confirms that NCYC2592 and M2 are ale strains (*Saccharomyces cerevisiae*), while W34/70 is a lager strain (*Saccharomyces pastorianus*). Furthermore, yeast strains differentiation were revealed through DNA fingerprint, which distinguishes all yeast strains evaluated and indicates that they are genetically distinct (Figure 1). These disclose the fact of yeast contains several mineral elements (including Pi, K, Mg, Cu, Zn, Fe, Mn, Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Li, Mo, Na, Ni, Pb, Rb, S, Se, Sr, Tl, U, V) (Table 4), which are essential for yeast growth, health, and survival [24]. Due to their genetic distinction, yeast strains contain different mineral profiles although the five more concentrated minerals in their content are K, P, S, Na, and Mg (Table 4/Figure 2).

Indeed, several physical, chemical, and biochemical transformations occur simultaneously in the yeast environment during alcoholic fermentation, which involves mineral ions as co-factors of several important anabolic and catabolic reactions [22]. Our results reveal that all yeast strains evaluated contain thirty-two mineral elements, which were divided into macro minerals (group 1 and 2) and micro minerals (group 3 and 4) (Table 4). Yeats *Saccharomyces cerevisiae* contain a range of macro minerals in the following order: K > P > S > Mg > Na > Ca [23]. Our findings reveal that all yeasts evaluated contain as macro minerals including P, K, Mg, Ca, Fe, Cu, Zn, Mn, S, Na, Al, B, Ru, and Sr (Table 4). However, each yeast strain contains different concentration of all mineral content identified. NCYC2592 (**K > P > S >**

Na > Mg > Zn > Ca > Mn > Fe > Cu), W34/70 (**K > P > Na > S> Mg** > Mn > Zn > Ca > Fe > Ru), and M2 (**P > K > Na > S > Mg** > Ca > Zn > Mn > Fe > Ru) contain a different concentration of the macro minerals but K, P, S, Na, Mg Zn, Ca, Mn, and Fe are the nine main mineral contained in yeast cell structure (Table 4). Therefore, mineral composition endorse that all yeast strains evaluated are genetically distinct. Due to its genetic distinction, all yeasts evaluated can be distinguished by the type of assimilated carbon source, growth temperature, and mineral content.

Macro minerals such as inorganic-phosphate, potassium, magnesium, zinc, iron, copper, and manganese are essential inorganic elements that influence cell metabolic activities as co-factors [17, 24-25]. Sugars are essential for fermentation, and amino acids (nitrogen source) stimulate carbon consumption and biomass production [26]. However, essential inorganic elements contribute to growth, fermentation performance and final product quality (flavors) [15, 17]. These inorganic elements, as charged ions, may establish chemical gradients such as adenosine triphosphate (ATP) synthesis into mitochondria via oxidative phosphorylation and/or secondary transport [24]. Lager strains (W34/70) showed the higher concentration of lithium and sodium than ale strains (NCYC2592 and M2), which may be related to yeast strains' natural capacity for potassium replacement [15, 24-25]. *Saccharomyces cerevisiae* (NCYC2592 and M2) contain a higher concentration of phosphorus, potassium, magnesium, and calcium than *Saccharomyces pastorianus* (W34/70). In contrast, W34/70 contains a higher concentration of iron, zinc, and manganese than NCYC2592 and M2. These may explain why *Saccharomyces cerevisiae* can grow under higher temperature conditions than *Saccharomyces pastorianus* (Figure 2). Correlating yeasts growth temperature and mineral composition reveal that yeasts that contain more potassium a higher concentration of (K), B (Boro), Al (Aluminium), V (Vanadium), Ba (Barium), and TI (Thallium) in their composition, tends to increase their temperature resistance (Figure 2). These cited minerals show correlation values with growth temperature tested greater than 0.8. Moreover, a part of yeast structure, yeast requires minerals for its metabolic activities [17, 24]. The generation of yeast metabolites is related to the presence of inorganic phosphate, potassium, and magnesium, which establishes a paradigm for future studies of yeast enzyme activation [17]. Magnesium, potassium, and phosphorus showed their highest concentrations in *Saccharomyces cerevisiae* strains (NCYC2592 and M2) than in *Saccharomyces pastorianus* (W34/70) (Table 4). M2 contains the highest alkali metals content (including calcium, rubidium and caesium) than other two strains evaluated, which may explain the high flocculation characteristics of this strain (bottom-fermentation). All yeast strains evaluated in this study differ its environmental survival capacity (including carbon source, and growth temperature), and its mineral content due to its physiological differences.

Food waste and protein for food and feed are among the worldwide concerns due to the population growth and market demands [17]. Protein produced by single cell protein by semi-solid state fermentation are also well-known. However, a large quantity of yeast cells are generated from breweries, wineries, and distilleries, which have been mostly treated as waste. Although countries such as United Kingdom utilise the brewing yeast to produce a food spread based on yeast extract; however, the majority of yeasts generated from breweries, wineries, and distilleries are still unused. Moreover, all the studies using yeast as single cell protein utilised bakers' yeasts. Our results show that all strains evaluated can be used for single cell protein generation using fruit waste because the majority of carbohydrates present in fruit waste are glucose, fructose, and sucrose, which the strains evaluated grew on well. These strains cannot be applied on subtracts, which contains high concentration of xylose because they cannot consume this carbohydrate (Table 2). W34/70 can be applied on raw materials that contains high concentration of rhamnose and melibiose (Table 3). Yeast strains such as NCYC2592 can grow at 40 °C, which is an important information for fermentation processes conducted during short period of time (up to 24 h) because this process tends to be controlled at temperatures between 28-32 °C. However, during fermentation, yeasts generates energy, which increases this temperature up to 38 °C. Therefore, identify yeast strains, which survives at high temperatures is important for industries. This study assessed three-yeast strains very well use by breweries, wineries, and distilleries; and our results obtained the genetic/physiologic potential these strains. Also, our results indicate that yeast from breweries, wineries, and distilleries can be applied as single cell proteins using several different food waste by semi solid state fermentation. Furthermore, each yeast strain can be applied for a different food waste to guarantee its optimum processing conditions. Future studies to evaluate and optimise the best yeast-cell for each fruit waste are in need. In the future, industries can reduce yeasts waste and fruit waste; and increase protein source, therefore, this knowledge will contribute for food waste utilisation, sustainable food processing, world's protein demand, and protein for vegetarian market as advocated by Ribeiro-Filho et al. [2].

5. Conclusion

The assess of three-yeast strains very well use by breweries, wineries, and distilleries was evaluated and we did obtain their genetic/physiologic characteristics and their potential to be utilised in other bioprocesses. All strains evaluated grow at 22, 25, and 30 °C. NCYC2592 and M2 can grow at 37 °C. NCYC2592 can grow at 40 °C. When inoculated on glucose, fructose, sucrose, maltose, all strains studied grow as a very intense growth (OD 0.8-1.0). NCYC2592 and M2 can present a very intense growth when inoculated on galactose and mannose. When inoculated on galactose and mannose, W34/70 presented an intense growth (OD 0.6-0.8). W34/70 presented a very intense growth on melibiose and an intense growth on rhamnose; NCYC2592 and M2 could not grow on these carbohydrates. DNA fingerprint confirmed their genetically differences. All strains evaluated contains third three minerals in their composition including Pi, K, Mg, Cu, Zn, Fe, Mn, Ag, Al, As, Ba, Be, Cd, Ca, Co, Cr, Cs, Li, Mo, Na, Ni, Pb, Rb, S, Se, Sr, Tl, U, V. NCYC2592 and M2 have a more similar mineral composition.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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