FUNGAL TERRITORY
2021, VOL. 4, NO. 4, 1-3

OPEN ACCESS
SCICELL

REGULAR ARTICLE

PHYSICOCHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES OF MANGO (*MAGNIFERA INDICA* L.) SEED KERNEL AND PEEL OILS

*Zekeria Yusuf *, Alemtsehay Malede, Mulugeta Desta, Megersa Idris, Sultan Seyida*

Address (es):
Haramaya University, Ethiopia

*Corresponding author: zakoyusuf@yahoo.com

https://doi.org/10.36547/ft.374

ABSTRACT

The peel and kernels mango (*Mangifera indica* L.) processing by products can be used as a source of valuable products. Therefore, the present study was attempted to study physicochemical properties, antioxidant and antimicrobial activities of mango seed kernel and peel wastes. The result of physicochemical properties indicated that significantly higher oil yield (38.75±1.77), specific gravity (0.86±0.04), acid value (2.66±0.20) and free fatty acid value (1.34±0.12); and higher DPPH (16.70±0.70) antioxidant activities were recorded for mango seed oil extract. However, significantly higher hydrogen peroxide scavenging activity (HPSA, 31.10±1.70) and ascorbic acid (43.00±2.73) were recorded for fruit peel oil extract. Stronger antibacterial activity with maximum zone of inhibition (16.50 mm), minimum inhibitory concentration MIC (0.10µl/ml) and corresponding minimum bactericidal concentration MBC (0.20 µl/ml) was recorded for seed oil extract against *S. aureus*. Stronger antifungal activity with maximum zone of inhibition (16.47 mm), MIC (0.05 µl/ml, the least value) and MFC (0.10 µl/ml) for seed oil extract against *C. albicans*. It can be concluded from the results of present study that seed oil extract was found to be more effective antioxidant and antimicrobial potential than peel oil extract in mango *(M. indica* L.*)*

Keywords: Antibacterial potential, Diameter of zone inhibition, Free radical scavenging activities, MBC, MFC, MIC

INTRODUCTION

Mango (*Magnifera indica* L.) belonging to Anacardiaceae family, is known for its attractive color, delicious taste, good flavors **(Pott *et al.*, 2003)**. During the processing of mango for pulp, the stone containing seed kernel contributing 15-20 % of total fruit weight is generated as by-product. The mango seed kernel contains protein, carbohydrates, high oleic lipids, minerals, crude fiber, ash, various bioactive compounds. The mango seed kernel contains almost 15% edible oil **(Akinyemi *et al.*, 2015)**. The variation in oil content can be due to cultivar differences, soil type, ripening stage, the harvesting time and the extraction method of oil used **(Arogba, 1997; Yamoneka *et al.*, 2015)** and difference in climatic conditions of their geographical locations **(Sani, 2014)**. Mango oil is good for baby creams, suncare balms, cosmetic, soap industry and within other moisturizing products **(Kittiphoom and Sutasinee, 2010)**. Depending on the cultivar, the mango seed represents from 10 % to 25 % of the whole fruit weight **(Ahmad *et al.*, 2007)**. The kernel inside the seed accounts for 45 % to7 5% of the seed and about 20 % of the whole fruit. Domestic consumption and industrial processing of the fruits produce huge amount of mango seed kernel and peels by products. The processing of such byproduct wastes into valuable products helps in resolving the problems of raw materials, energy, environmental pollution, and drug resistances.
Therefore, the present study was attempted to study physicochemical properties, antioxidant and antimicrobial activities of mango seed kernel and peel wastes.

MATERIALS AND METHODS

Plant material and extract preparation

The experiment was conducted in Molecular Biology and Biotechnology Laboratory, Haramaya university. The mango fruit sample was collected from Home Garden, Bahirdar district, Ethiopia. The fruit samples were manually washed with distilled water and residual moisture was evaporated at room temperature. Thereafter, the seeds and peel samples were chopped and ground in a grinder for 2 min, the process was stopped for 15 sec to avoid heating of sample. The oil extraction was done in Soxhelt apparatus using hexane as a solvent. Then, physicochemical properties of the oil were done based on determination of oil content, specific gravity, acid value, free fatty acid and peroxide value, and the antioxidant activities were based on DPPH and hydrogen peroxide free radical scavenging activities, and ascorbic acid as per the standard procedure described by **AOAC (1990)**.

Antimicrobial activity of the oil extracts

The antimicrobial experiment was arranged as 2 x 1 x 4 [2 source extracts: seed and peel oil extracts of Mango (*Mangifera indica* L.) at three concentration levels, 1 solvent system i.e. hexane, 4 test organisms (2 bacteria: *E. coli* (gram negative), and *Staphyllococcus aureus* (gram positive), and; two fungi (*Aspergillus niger* and *C. albicans*)] completely randomized factorial design in three replications. The test pathogens were obtained from Ethiopian Institute of Food and Health, Addis Ababa, Ethiopia. The fungal and bacterial pathogens were subcultured and maintained on Potato Dextrose Agar (PDA) and Nutrient Agar, respectively. Thenceforth, the fungal and bacterial cultures were incubated for 72 h at 27 ºC and for 18-24 h at 37 ºC, respectively.

Media Preparation and Standardization of Inoculum

Nutrient Agar (NA), Potato Dextrose Agar (PDA), and Muller Hinton agar (MHA) was used for sub-culturing of bacterial test organism, fungal test organism, and determination of antimicrobial activities, respectively. These media were prepared and sterilized using an autoclave according to the manufacturers' instructions. Two to three bacterial colonies on the plate were picked up with a sterile inoculating loop and transferred into a test tube containing sterile normal saline and vortexes thoroughly. The spores of the test fungi were harvested by washing the surface of the fungal colony using 5mL of sterile saline solution. This procedure repeated until the turbidity of each bacterial and fungal spore suspension matched the turbidity of 0.5 McFarland Standards as described by the Clinical Laboratory Standards Institute **(CLSI, 2012)**. The resulting suspension was used as inoculums for the test pathogen in the antimicrobial susceptibility test.

Disc diffusion Method

The discs of 6 mm diameter were prepared from sterile filter paper cut into small, circular pieces of equal size by a perforator and then impregnated each of them was impregnated with 0.01 ml of the prepared test extract ethyl acetate solution. The extract impregnated discs were placed onto MHA plates evenly inoculated with test pathogens **(Abdel Wahab and Gismalla, 2017)**. Following this step, the impregnated discs were dispensed onto the surface of the inoculated agar plates using sterile forceps **(CLSI, 2015)**. Discs of commercial ampicillin (1µl/disc) and ketoconazole (1µl/disc) were used as positive controls for bacterial and fungal pathogens, respectively and distilled water impregnated discs were used as negative controls. Then the MHA plates were sealed with parafilm and incubated at 37 ºC for 24 h and 27 ºC for 72 h for bacterial and fungal pathogens, respectively. The diameters of the zone of inhibition around each disc were measured to the nearest millimeter along two axes (i.e. 90° to each other) using a transparent ruler and the means of the two readings were be recorded.

Determination of Minimum Inhibitory Concentration (MIC)

The oil extracts that showed significant antimicrobial activity in the antimicrobial activity tests were selected for determination of MIC based on broth dilution method followed by **Mousavi *et al.* (2015)** with slight

modifications. In broth dilution method, two milliliters of nutrient broth and potato dextrose broth for bacteria and fungi respectively were added into all test tubes and 0.1 ml of the prepared concentration of each oil extract were mixed with the nutrient broth and potato dextrose. Thereafter, standardized inoculums of 0.1ml of the respective test pathogens were dispensed into the test tubes containing the suspensions of the broth and the oil extract. Then, all test tubes were properly corked and incubated at 37 °C for 24 h for bacteria and 27 °C for 72 h for fungi. After that, they were observed for absence or presence of visible growth. The lowest concentration at which no visible growth of organisms was regarded as the MIC. The experiment was carried out for each test organism in triplicates.

Determination of minimum bactericidal (MBC) and fungicidal concentrations (MFC)

For the determination of the MBC and MFC, fresh nutrient agar and potato dextrose agar plates were inoculated with a loop taken from each of the broth cultures that showed no growth in the MIC tubes. That is MBC/MFC values were determined by subculturing from respective MIC values. Since antibacterial agents are usually regarded as bactericidal if the MBC is no more than four times the MIC **(CLSI, 2015)**. MBC/MFC is the amount of the extract that kills microbial growth. While MBC assay plates were incubated for 48 h, MFC assay plates were incubated for 3 days. After the incubation periods, the lowest concentration of the extract that did not allow any bacterial or fungal growth on solid medium was regarded as MBC and MFC for the extract **(CLSI, 2012)**.

Statistical analysis

The experimental data were analyzed using SAS version 9.2. **(SAS, 2011)** to investigate statistical significance between the different oil quality parameters. Differences between means were considered statistically significant at *P<0.05*.

RESULTS AND DISCUSSION

Physicochemical properties of mango seed and peel oil extracts

The physicochemical properties of mango seed and peel oil extracts were assessed based on physicochemical parameters like oil content, specific gravity, acid value, free fatty acids, and peroxide values as in Table 1.

Table 1 Physicochemical properties of mango seed and peel oil extracts

Oil extract	Oil yield	Spgr	ACV	FFA	PV
Seed	38.75±1.77^{a}	0.86±0.04^{a}	2.66±0.20^{a}	1.34±0.12^{a}	1.10±0.25^{b}
Peel	31.00±1.41^{b}	0.69±0.03^{b}	1.54±0.01^{b}	0.78±0.10^{b}	2.20±0.28^{a}

Legend: Means followed by same letter within a column were not significantly different at *P<0.05* probability level based on LSD (Least Significance difference) test. Small letters: significance within column; Spgr: specific gravity; ACV: acid value; FFA: free fatty acids; PV: peroxide value

It was observed that significantly higher oil yield (38.75±1.77), specific gravity (0.86±0.04), acid value (2.66±0.20) and free fatty acid value (1.34±0.12) for mango seed oil extract. However, significantly higher peroxide value (2.20±0.28) was recorded for peel oil extract. Similar study was conducted by Abdelaziz (2018) who reported the characteristics of mango kernel meal oil The Specific gravity at 24° C, Reflective Index, and Iodine value (g/100 g oil) were 0.89, 1.58 and 46.0, respectively.

The peroxide values for mango seed (1.10) and peel (2.20) of mango oil extract obtained in the present study were lower than that expected of rancid oil which ranges from 20.00-40.00 mg/g oil **(Ishida *et al.*, 2000)**. Generally, in the fresh oil, the peroxide value should be less than 10 mg/g oil **(Kittiphoom and Sutasinee, 2013)**. High peroxide values are associated with higher rate of rancidity **(Sani, 2014)**. Acid value for mango seed oil (2.66) and peel oil (1.54mg KOH/g), indicates that the oils were edible because it falls within the recommended codex of 0.6 and 10 for virgin and non-virgin edible fats and oil, respectively **(Olajumoke, 2013)**.

Antioxidant activities of oil extracts of mango seed and peel oil extracts

The antioxidant activities of mango seed and peel oil extracts was evaluated based on DPPH and hydrogen peroxide free radical scavenging activities and ascorbic acid content as in Table 2. There was no significance difference in DPPH between kernel seed and peel oil extracts even though, it was higher for seed oil extract (16.70±0.70). Significantly higher hydrogen peroxide scavenging activity (HPSA, 31.10±1.70) and ascorbic acid (43.00±2.73) were recorded for mango fruit peel oil extract. The higher DPPH value indicates higher antioxidant activities and the presence of higher essential omega-3 fatty acids in mango seed oil extract. The DPPH radical scavenging activity of hexane extract was higher than that of ethanol extract because hexane more extracting other antioxidant components soluble in oil, such as β-carotene and vitamin E (tocopherols and tocotrienols) **(Masud *et al.*, 2018).**

Table 2 Antioxidant activities of mango seed and peel oil extracts

Oil extract	DPPH	HPSA	AA
Mango seed	16.70±0.70^{a}	12.65±0.92^{b}	14.74±2.98^{b}
Mango peel	14.05±1.20^{a}	31.10±1.70^{a}	43.00±2.73^{a}

Legend: Means followed by same letter within a column were not significantly different at *P<0.05* probability level based on LSD (Least Significance difference) test. Small letters: significance within column; DPPH: 2, 2- diphenyl-1-picrylhydrazyl; HPSA: hydrogen peroxide scavenging activity; AA: ascorbic acid

Antimicrobial Activities of Mango (Mangifera indica L.) seed and peel oil extracts

The antimicrobial activities based on diameter of inhibition zone for *Mangifera indica* L. seed and fruit peel oil extracts were shown in Table 3. Significance differences were recorded for both seed and peel oil extracts at different concentration levels. The mean zone of inhibition at highest concentration (3 µl/ml) against bacterial test pathogens ranged from 13.90±0.36 mm to16.50±0.45 mm, while 12.67±0.40mm to 16.47±0.50 mm against fungal test pathogens. Stronger antibacterial activity with maximum zone of inhibition (16.50 mm) at highest dose (3 µl/ml) was recorded for seed oil extract against *S. aureus* while the weaker antibacterial activity (13.90 mm) was observed for peel oil extract against *E. coli* indicating that *S. aureus* (gram positive) was more susceptible to the oil extract than *E. coli* (gram negative). Thus, seed oil has exhibited more antibacterial potential than peel oil in mango (*Mangifera indica* L.).

Table 3 Antimicrobial Activities oil extracts from mango seed and peel oil extracts as mean diameter of zone of inhibition against test pathogenic microorganism

Test microorganism	Oil extract	Concentration of the oil extract (v/v)			Ciprofloxacin (1µl/ml)
		1µl/ml	2µl/ml	3µl/ml	
E. coli	Seed	11.67±0.76aC	12.73±0.64aC	15.03±0.45bB	18.56±0.40aA
	Peel	11.00±0.50aBC	12.50±0.45aBC	13.90±0.36cB	18.60±0.40aA
S. aureus	Seed	11.17±0.29aC	12.30±0.26aC	16.50±0.45aB	18.60±0.36aA
	Peel	11.67±0.76aC	13.33±1.26aBC	14.83±0.76bcB	18.87±0.32aA
					Fluconazole (1µl/ml)
C. albicans	Seed	12.43±0.40aD	14.33±0.76aC	16.47±0.50aB	18.17±0.26bA
	Peel	11.83±0.76aC	13.83±0.57aB	14.87±0.71bB	19.00±0.50aA
A. niger	Seed	0.00±00cD	9.83±0.76cC	13.70±0.26cB	18.50±0.50abA
	Peel	10.73±0.25bC	12.00±0.50bB	12.67±0.40dB	18.83±0.29abA

Legend: Means followed by same letter within a column were not significantly different at *P<0.05* probability level based on LSD (Least Significance difference) test. Small letters: significance within column; capital letters: significance across row. *E. coli*: *Escherichia coli*; *S.aureus*: *Staphylococcus aureus, C. albicans*: *Candida albicans, A. niger*: *Aspergillus niger*

By contrast, stronger antifungal activity with maximum zone of inhibition (16.47 mm) was recorded for seed oil against *C. albicans* as the weaker antifungal

activity with minimum zone of inhibition (12.67 mm) was observed for peel oil against *A. niger* suggesting seed oil extract is more effective antifungal potential than peel oil extract in *M. indica*. Similar study was conducted by **Vega-Vega *et al*. (2013)** who demonstrated a significantly higher total antioxidant capacity, phenolic content, and antimicrobial activity of mango byproducts (seed and peel) than of the edible portions.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), minimum fungicidal concentration (MFC) of oil from *Mangifera indica* L. seed and peel oil extracts

The efficacy of *Mangifera indica* L. seed and peel oil extracts against pathogenic microbes was evaluated by MIC, MBC and MFC as in Table 4. The oil extracts from seed kernel has exhibited strongest antibacterial activity with MIC (0.10 µl/ml) and corresponding MBC (0.20 µl/ml) against *S. aureus* while the weakest antibacterial activity with MIC (0.75 µl/ml, the largest value) and MBC (1.00 µl/ml) was recorded for peel oil against *E. coli* indicating that *S.aureus* is more susceptible to the oil extract than *E.coli*, and also indicating seed oil possesses stronger antibacterial potential than peel oil in mango fruit.

Table 4 MIC, MBC, and MFC of *Mangifera indica* L. seed and peel oil extracts

Test microorganism	Oil extract	MIC (µl/ml)	MBC/MFC (µl/ml)
E. coli	Seed	0.20	0.25
	Peel	0.75	1.00
S. aureus	Seed	0.10	0.20
	Peel	0.40	0.75
A. niger	Seed	0.25	0.50
	Peel	0.50	1.00
C. albicans	Seed	0.05	0.10
	Peel	0.20	0.25

CONCLUSION

Based on the results of physicochemical properties of mango seed kernel and peel oils, it could be concluded that the oil extract could be become valuable resource to produce high value of vegetable oil. The oil extracted with hexane has better quality. The results of present study provide useful information for edible oil and food industry, due to biological activities as antioxidant and antimicrobial activities.

Authors' contribution

Zekeria Yusuf: initiation and design of the study, Lab experiment, data analysis; Alemtsehay malede and Megersa Idris: Lab experiment, data collection, and write up of the document; Sultan Seyida and Mulugeta Desta: Analysis and interpretation of data. All authors contributed to drafting the article and revising it critically for important intellectual content.

Acknowledgements

The authors are grateful to Haramaya University Research Office for their financial support and Laboratory facility.

Funding

This project was funded by Haramaya University Research grant, under project code: HUIF_2019_06_01_56

Availability of data and materials

The data materials will be available on request.

Ethics approval and consent to participate

The ethical approval is not applicable for this manuscript since it has no animal experiment according to Haramaya University's ethical committee.

Conflict of Interest

The authors declare no conflict of interest.

REFERENCES

A.O.A.C. (1990). Official Methods Of Analysis, 15th Ed. Association Of Official Chemists' Washington Dc.

Abdelaziz Sa. 2018. Physico Chemical Characteristics Of Mango Kernel Oil And Meal. Middle East Journal of Applied Sciences. Volume : 08 Pp 01-06.

Ahmed EMA, Darwish SM, Ayad EHE, El-Hamahmy RM 2007. Egyptian mango by product 1, compositional quality of mango seed kernel. Food Chem. 103(4): 1134-1140. https://doi.org/10.1016/j.foodchem.2006.10.017

Arogba SS. 1997. "Physical, chemical and functional properties of Nigerian mango (Mangifera indica) kernel and its processed flour". J SCI FOOD AGRIC, Vol.73, No.3, pp. 321- 328, 1997. https://doi.org/10.1002/(SICI)1097-0010(199703)73:3%3C321::AID-JSFA722%3E3.0.CO;2-4

Yamoneka J, Malumba P, Blecker C, Gindo M, Richard G, Fauconnier M, Lognay G, Danthine S. 2015 "Physicochemical properties and thermal behaviour of African wild mango (Irvingia gabonensis) seed fat". LWT-food science and technology,Vol. 64, No.2, pp. 989-996. 2015. https://doi.org/10.1016/j.lwt.2015.06.035

Sani I. 2014. "Soxhlet extraction and physicochemical characterization of Mangifera indica L. Seed kernel oil". Research and Reviews: J Food Dairy Tech, Vol. 2, No.1, pp. 20- 24.

Kaur J., Rathinam X., Kasi M., Leng K. M., Ayyalu R., Kathiresan S., Subramaniam S. 2010. Preliminary Investigation On The Antibacterial Activity Of Mango (Mangifera Indica L: Anacardiaceae) Seed Kernel. Asian Pacific Journal Of Tropical Medicine, 707-710. https://doi.org/10.1016/S1995-7645(10)60170-8

Vega-Vega V., Silva-Espinoza Ba., Cruz-Valenzuela M.R., Bernal-Mercado At., González- Aguilar Ga., Ruíz-Cruz S., Moctezuma E., Siddiqui Mdw, Ayala-Zavala Jf. 2013. Antimicrobial And Antioxidant Properties Of Byproduct Extracts Of Mango Fruit. Journal of Applied Botany And Food Quality 86, 205 - 211.

Abdel Wahab M., Gismalla M. 2017. Antimicrobial Activity Of Aqueous Methanol Extracted Rigla (Portulaca Oleracea Linn). M.Sc Thesis. University Of Gezira. http://repo.uofg.edu.sd/handle/123456789/781

Akinyemi S. O. S, Akin-Idowu, P. E., Oduntan, O. O., Egbekunle, K. O. 2015. "Chemical Composition Of The Seed Kernel Flour Of Some Mango (Mangifera Indica L.) Varieties". J. Biol. Chem. Research, 32(1): 160-173.

Clinical And Laboratory Standards Institute (Clsi) (2015). Document M45. Methods For Antimicrobial Dilution And Disk Susceptibility Of Infrequently Isolated Or Fastidious Bacteria; Approved Guideline. Third Edition. Clsi, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898, Usa.

Clinical And Laboratory Standards Institute (Clsi, 2012). Performance Standards For Antimicrobial Disk Susceptibility Tests; Approved Standards – Eleventh Edition. Clsi Document M02-A11. Clinical And Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pa, 19087 Usa, 2012.

Ishida H, Suzuno H, Sugiyama N, Innami S, Tadokoro T, Maekawa A.2000 "Nutritive Evaluation On Chemical Components Of Leaves, Stalks And Stems Of Sweet Potatoes (Ipomoea Batatas Poir)". Food Chem, Vol,68, No.3, Pp. 359-367. https://doi.org/10.1016/S0308-8146(99)00206-X

Kittiphoom S, Sutasinee S. 2013. "Mango Seed Kernel Oil And Its Physicochemical Properties". International Food Research Journal, Vol.20, No.3, Pp. 1145-1149.

Masud F., Mahendradatta M., Laga A., Zainal Z. 2018. Physicochemical Properties And Fatty Acid Composition Of Mango Seed Kernel Oil. International Journal Of Engineering Sciences & Research Technology. 7(1): 361-369. https://doi.org/10.5281/zenodo.1147523

Mousavi S.M., Bagheri G., Saeidi S. 2015 Antibacterial Activities Of The Hydroalcoholic Extract Of Portulaca Oleracea Leaves And Seeds In Sistan Region, Southeastern Iran. Int J Infect. 2(2):E23214. https://dx.doi.org/10.17795/iji-23214

Olajumoke Oe. 2013. "Extraction And Characterization Of Vegetable Oil From Mango". Iosr Journal Of Applied Chemistry, Vo.5, No.3, Pp. 6-8. https://dx.doi.org/10.5281/zenodo.1147523

Pott M., Neidhart S., Muhlbaur W., and Carle R. (2003). Quantitative determination of B-carotene stereoisomers in fresh, dried and solar dried mongoes (Mangifera indica L). J. of Agric and Food chem. 51: 4527-4531. https://doi.org/10.1021/jf034084h

SAS Institute (2011). SAS enterprise guide, Version 9.2. SAS Inst., Cary, NC, USA.

FUNGAL TERRITORY
2021, VOL. 4, NO. 4, 4-9

OPEN ACCESS

SCICELL

REGULAR ARTICLE

ISOLATION OF WILD YEAST FOR POTENTIAL USE IN BEER PRODUCTION

Priscila Treviño-Aguilar[1], Myriam Elias-Santos[1], Claudia López-Alvarado[1], Benito Pereyra-Alférez[1], Jorge Hugo Garcia-Garcia[*1]*

Address (es):
[1] Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Instituto de Biotecnologia, Pedro de Alba s/n, 66451, San Nicolas de los Garza, Nuevo León, 8183294000 ext. 6438.

*Corresponding author: jorge.garciagr@uanl.edu.mx

https:/doi.org/10.36547/ft.367

ABSTRACT

There is a limited amount of yeast strains that are currently used in industrial beer brewing. Wild yeasts could provide an alternative to common domesticated brewer's yeasts by offering a new range of sensory characteristics and improved performance in harsh brewing conditions such as high gravity and high ethanol concentration in wort. High gravity brewing is practical and profitable as it increases production capacity, therefore reducing investment and energy costs. Exploiting the existing natural diversity could lead to finding superior industrial yeasts as well as a better understanding of biodiversity. The aim of the present study was to determine if wild yeast strains isolated from the ecosystem at a regional level (Northeast Mexico) showed favorable characteristics in the brewing process. Sixty-three yeast isolates were obtained from diverse samples including flowers and fruits. Five isolates were selected after three rounds of beer fermentation based on their sensory characteristics. Tests to assess growth over time, flocculation potential, ethanol yield, osmotolerance and ethanol tolerance were applied to two reference yeasts (commercial beer brewing strains) and to the selected isolates of interest which were identified as *Saccharomyces cerevisiae* by *MEX67* amplification and ITS sequencing. The results indicated that the selected wild isolates exhibit characteristics comparable to commercial reference strains in terms of growth and stress tolerance.

Keywords: wild yeasts, beer, *Saccharomyces cerevisiae*

INTRODUCTION

Beer is generally made from four main ingredients: water, barley malt, hops, and yeast. All of them are involved in beer's final flavor, including yeast, which is responsible for the production of diverse flavor compounds and ethanol **(Mosher, 2009)**. Beer fermentation is done mainly by members of the *Saccharomyces* genus. There are few yeast strains that are used industrially in the brewing process, they can be grouped in two main classes: Ale (*Saccharomyces cerevisiae*) and Lager strains (*Saccharomyces pastorianus*). Yeasts represent a very diverse group of organisms and even strains that are classified as the same species often show a high level of genetic divergence **(Steensels *et al.*, 2014)**. Lager strains are a natural interspecific hybrid organism between *S. cerevisiae* and *S. eubayanus*. This hybrid obtained improved growth rates and biomass yields at low temperature from its *S. eubayanus* subgenome and the ability to consume maltotriose from *S. cerevisiae* **(Hebly *et al.*, 2015)**. Commercial strains of *S. cerevisiae* have shown specific selection for stress tolerance, sugar utilization and flavor production **(Parapouli *et al.*, 2020)**. However, concentrated beer production may have caused a reduction in the number of strains used for brewing, thus it is relevant to explore opportunities to isolate or generate yeast variants that fulfill the interest for increased ethanol productivity, extensive substrate utilization and production of non-conventional compounds **(Huuskonen *et al.*, 2010; Cubillos *et al.*, 2019)**.

Some strategies that have been carried out to provide new yeast strains to the brewing industry include experimental evolution, mutagenesis, breeding, and yeast isolation from the wild. Isolating wild yeasts from a particular geographical region to make the most of its inherent biodiversity may provide novel flavors and can conform to the demand of non-genetically modified microorganisms and use of Generally Recognized as Safe (GRAS) substances. Recent efforts across the globe have contributed to the expansion of the brewing yeasts' reserve by sampling from forests and orchards. Depending on the isolation method utilized there has been varying success in finding new *Saccharomyces* strains or species from the wild **(Cubillos *et al.*, 2019)**. Although ubiquitous, yeast strains are commonly associated with sugar-rich environments. Wild yeasts are commonly isolated from sources like fruits, plants (roots, leaves, flowers, and exudates) and grains. Other strains are associated with soil, orchard ambient air and specific sources, such as fruit juice, honey, and flower nectar. This is convenient since it implies an inherent ability to grow in an environment of high sugar concentration **(Herrera *et al.*, 2009; Wang *et al.*, 2015)**. Wild yeasts most frequently associated with brewing are natural strains of *Saccharomyces* and *Brettanomyces* and, to a much lesser extent, *Candida* and *Pichia*, as well as other aerobic yeasts. During the production of Lambic beers, for example, wild strains of *Saccharomyces* and *Brettanomyces* are the main determinants of the sensory profile and the level of attenuation of beer **(De Keersmaecker, 1996)**. The use of wild yeasts in brewing, however, can lead to unpredictable fermentation results. Since they are undomesticated, they may be characterized by low fermentation yields and more sensitivity to ethanol stress, but usually provide a distinctive flavor, which could make them an adequate choice as a subsidiary fermentation agent. In addition, some may offer an advantage over commercial strains as they exhibit osmotolerance to variable degrees **(Wang *et al.*, 2015; Capece *et al.*, 2018)**. Some documented successful findings of wild yeasts capable of producing beer with sensory desirable characteristics include a strain of *B. anomalus* that was also an effective as starter culture in batches of multiple gallons of beer and a strain of *Saccharomyces cerevisiae* called YH166, which was isolated from an open fermentation in a vacant lot in the United States and gave beer a pleasant fruity aroma reminiscent of pineapple and guava **(Lentz *et al.*, 2014; Osburn *et al.*, 2017)**. However, it has also been found that some strains of wild yeasts may exhibit strong phenolic characters (plastic, medicinal and smoked), producing beers with a complex, spicy, fruity character and aromas that could be considered undesirable **(Bokulich and Bamforth, 2013)**.

Yeast metabolism plays a vital role in flavor compound production. Alcohols, esters, organic acids, carbonyl compounds and sulphur compounds are some of the metabolites of importance for beer's flavor profile **(Olaniran *et al.*, 2017)**. Volatile esters are responsible for beer's fruity character, they can have a synergistic effect with one another and, as trace compounds, a minimal change in their concentrations can dramatically affect the taste of beer **(Verstrepen *et al.*, 2003)**. The concentrations of these compounds can be affected by the yeast strain of choice (genetics), nutritional factors, environmental conditions during fermentation, among other factors **(Nedovic *et al.*, 2014)**.

Intensification of the industrial brewing process has also created a need for yeast strains that can excel in high and very high gravity brewing (Gibson, 2011). Most commonly, beer wort (water with dissolved sugars from malted barley) has a sugar concentration of 10 to 12 degrees Plato (°P), in some cases also high gravity wort is used (14 to 17 °P), producing beer with higher concentrations of ethanol which is subsequently diluted to a normal drinking strength. Therefore, the use of high gravity brewing reduces investment costs, energy consumption and labor costs. A very high gravity wort is also applicable (18 to 25°P) to the brewing process representing an even greater economical and yield advantage **(Huuskonen *et al.*, 2010)**.

The approach of this study was to focus on the possibility that the natural biodiversity found in a geographical region might offer an opportunity to isolate novel yeasts with favorable characteristics for industrial beer production.

MATERIALS AND METHODS

Sample collection and isolation of wild yeast strains

In order to perform a pre-selection of wild yeasts that were suitable for brewing (ability to grow on beer wort nutrients), a barley malt culture medium (BMM) with a content of 6 ± 2 Brix degrees (°Bx) was used. In all cases the wort was brought to room temperature (25 °C) before measuring Brix degrees with a PAL-1 pocket refractometer (ATAGO, Tokyo, Japan). The BMM medium was prepared by mashing 150 g of pale malt 2 Row (Malteurop, Milwaukee, WI, USA) for every liter of water for 60 minutes at 66 °C and then autoclaving at 12 1°C for 15 minutes at 15 psi. Barley malt agar medium (BMA) was also prepared by adding 20 g of bacteriological agar per liter before autoclaving.

Samples of flowers, tree bark, fruits, fruits skin, leaves and soil from different orchards of three north east Mexican states (Nuevo Leon, Durango and Coahuila) were aseptically placed in sterile falcon tubes containing 40 mL of sterile BMM. Air samples from the same locations were collected in triplicates by exposing BMA plates to air for 30 minutes.

All tubes were incubated at 25 °C for 5 days as a yeast enrichment step. Then the liquid cultures of all samples were serially decimally diluted using sterile 0.85% (w/v) NaCl solution and were spread onto BMA plates in triplicates and they were incubated at 25 ± 2 °C for 3 days. Cells of every colony type were isolated by repetitive streak plating, then morphological distinction of yeast cells was performed by observing them under a microscope at 1000X magnification. Pure cultures were maintained on 1% yeast extract, 2% peptone and 2% dextrose (YPD) agar and liquid medium and glycerol stocks of the isolates were prepared and stored at -40 °C.

Screening of yeast isolates

The wild yeast isolates of interest in this study were selected from all the isolates obtained based on their ability to ferment beer wort and produce beer with a pleasant sensory profile. To ensure that the isolates to be selected were able to maintain their vitality and continue to produce pleasant aroma and flavor compounds, 3 fermentation rounds were carried out in duplicates in 50 mL tubes, for 5 days each. After initially providing the respective pitching rate (0.75 million cells/mL/°P)14, the same bottom yeast sediment was reused each time. In all cases the same beer recipe was prepared by mashing 3.5 kg of 2-row pale malt and boiling 60 minutes with 30 g of cascade hops (specific gravity of around 1.035).

For each fermentation round, a tasting was carried out by a panel composed of 8 judges to characterize the product obtained by means of the 9-point hedonic scale test (affective test method) and a flash profile analysis (rapid descriptive analysis for sensory characterization).

The 9-point hedonic scale test was implemented as described by **Lim (2017)**, panelists were asked to indicate overall, how much they liked or disliked each sample and to fill out a ballot containing the following scale:

- Like Extremely
- Like Very Much
- Like moderately
- Like Slightly
- Neither Like nor Dislike
- Dislike Slightly
- Dislike Moderately
- Dislike Very Much
- Dislike Extremely

For flash profiling (FP), the method described by **Dairou and Siefferman (2002)** with some modifications was implemented. An attribute generation session was held first by presenting all the samples to the panelists one at a time. The samples were evaluated, and the perceived attributes were recorded (flavor and aroma attributes). Differently to the referenced method, the attributes were discussed by the judges and a consensus was reached (usually avoided in FP in order to save time). Afterwards, a sample rating session was held where a ballot containing the descriptors recorded before was given to every panelist to evaluate the samples once more, this time ranking them in order of intensity for each attribute generated. The following five-point system to rate attribute intensities, as recommended by **George and Laurie Fix (1997)**, was used:

1) Not detectable
2) Slightly detectable
3) Detectable, but not strong
4) Strong
5) Overpowering

All data was entered to a database afterwards and factor analysis was implemented for FP taking into account the mean intensity score for each attribute. Analysis was conducted in R **(R Core Team, 2021)** and all figures in this paper were produced using the packages ggplot2 and fortify **(Wickham, 2016; Tang *et al.*, 2016)**.

The reference yeasts in this study were included for comparison in each tasting, which consisted of the same beer wort recipe fermented by a commercial Ale type yeast (SafAle ™ S-04 Fermentis, Milwauke, USA) fermenting at room temperature (25 ± 2 °C) and a Lager type yeast (Saflager ™ S-189 Fermentis, Milwauke, USA) fermenting at 15 °C ± 2, according to the manufacturer recommendation. It was relevant to select reference yeasts from an industrial brewing yeast global manufacturer to be able to compare their fermentation properties to the wild isolates. In addition, the flavor profile of both references favored contrast during the sensory evaluation process. In the ale strain's case providing typical characteristics present in a large range of ales (balanced fruity and floral notes as described by the manufacturer). And in the lager strain's case allowing for a neutral flavor brew (mild flavor, low ester production) as opposed to the widely used strain Fermentis Saflager W-34/70, which provides more of a pronounced floral and fruity aroma.

The first tasting round consisted of sampling beer produced by all the yeast isolates collected fermented at 25 °C ± 2 °C for 5 days. For the second tasting round, each isolate was used in 2 different batches in duplicates at 2 different temperatures, 25 °C ± 2 °C (Ale type fermentation) and 15 °C ± 2 °C (Lager type fermentation), to determine which of them generated a more desirable final product and set it as its optimal fermentation temperature. Finally, the third tasting round consisted of using the isolates to produce beer only at their respective optimal fermentation temperature (referred to this way from this point on), which means that they received a higher hedonic score at this type of fermentation.

Identification of selected yeast isolates

Selected isolates were identified by obtaining their genomic DNA from overnight YPD medium cultures with 1 x 10^6 cells/mL by the method reported by **Osorio *et al.* (2009)**. Genomic DNA was used for ITS-5.8S amplification using primers ITS1 (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-CTTGGTCATTTAGAGGAAGTAA-3') for sequencing and restriction analysis using HaeIII **(Gardes and Bruns, 1993; McCullough *et al.*, 1998)**; species identification primers for *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. bayanus*, *S. arboriculus* and *S. cerevisiae* were used as reported by **Muir *et al.* (2011)**. *S. cerevisiae* is identified by gene MEX67 using primers ScerF2 (5'-GCGCTTTACATTCAGATCCCGAG-3') and ScerR2 (5'-TAAGTTGGTTGTCAGCAAGATTG-3'). The PCR products, 5 µL, were loaded on an agarose gel and electrophoresed at 70 V/cm2, stained with ethidium bromide and visualized. We used a 100 BP DNA ladder (Promega, Wisconsin, USA) and Lambda HindIII as molecular size markers (Promega, Wisconsin, USA). DNA bands for ITS-5.8S were purified using the Wizard SV 96 PCR Clean-Up System (Promega, Wisconsin, USA) and sequenced with the AB 3500 (Applied Biosystems, California, USA), to ensure the detection of the studied genes.

Growth kinetics: growth curve and flocculation potential assay

Growth performance over time of selected isolates was determined through a growth curve while also employing the commercial Lager type reference yeast for comparison (Saflager ™ S-189 Fermentis, Milwauke, USA) incubated at 15°C ± 2°C. The selected isolates and the reference yeast were inoculated in duplicate in tubes containing 50 mL of BMM, and then diluted (1:10) using sterile BMM, which was also used as blank. The growth curve was obtained by performing Optical density readings at 600nm (OD600) every 24 hours for 6 days with a SmartSpec 3000 spectrophotometer (Biorad, California, United States). The selected isolates were incubated at their optimal fermentation temperature and before performing every reading the culture was homogenized using a vortex mixer. Yeast cell counts were also performed using a Neubauer haemocytometer (Marienfeld). In all cases the respective pitching rate was calculated and provided (0.75 million cells/mL/°P) **(Fix, 1999)**.

After reaching the maximum growth point in all cases (no cell density increases in 24 hours), some fluctuation in the measured absorbance levels was observed. Thus, the residual absorbance of the yeast suspension throughout fermentation was investigated (residual absorbance before and after flocculation and settling). This method is described as an adequate parameter to characterize yeast flocculation potential, usually involving agitation, and resettling before measuring absorbance, and can also be combined with a direct observation of floc formation in the yeast culture **(Kihn *et al.*, 1988; Vidgren and Londesborough, 2011)**. However, the approach taken in this study was modified in accordance with the static fermentation method, which is considered to be an *in vivo* style test because it is carried out under conditions more closely akin to the static fermentation conditions encountered in a typical brewery (Stewart, 2018). The yeasts of interest were inoculated in 450 mL of BMM in 500 mL glass flasks and taking OD readings (600nm) every 24 hours for 10 days using the same method described before excepting without homogenizing the culture. This assay also served as a screening method

High gravity and ethanol tolerance assay

The selected isolates' tolerance to adverse fermentation conditions was determined by growing yeasts in culture medium simulating such conditions and then performing viable cell count by plate dilution method over time. The conditions tested were high glucose and high ethanol concentrations. The isolates of interest and both commercial reference yeasts (Lager type and Ale type) were inoculated in duplicates in tubes containing 10 mL of sterile YPD medium enriched with different glucose concentrations: 12°P, 17°P and 25°P, and ethanol concentrations: 4%, 8%, 12% and 16%. In all cases the tubes were inoculated with $9x10^6$ cells/mL and yeasts were incubated at their optimal fermentation temperatures (15 ± 2 °C or 25 ± 2 °C). A control was used in all cases (2°P, no added ethanol). Tubes with 0.9 mL of sterile 1M NaOH buffer were used to make dilutions (1:10 to 1:10,000,000) of the yeast cultures which were inoculated in petri dishes with YPD agar medium, and were left to grow for a period of 3-4 days at 25 °C ± 2 °C. The plates that showed growth in the range of 10 to 150 colonies were used to calculate the CFU/mL by multiplying the number of colonies by the dilution factor. The viable count by plate dilution method was repeated thrice. Once 24 hours after inoculating the tubes (T0). Once again at the yeasts' maximum growth point (T1, 4 days after T0) and then the last measurement was conducted at its stationary phase (T2, 9 days after T0).

To simply monitor sugar consumption, Brix degrees decline over time in the enriched growth medium of all cultures was also measured using a PAL-1 pocket refractometer (ATAGO, Tokyo, Japan). Readings of the medium's initial °Bx, as well as those of 5, 8, 15 and 25 days after the initial time were taken in triplicates.

Ethanol yield estimation assay

Selected isolates and both commercial reference yeasts (Ale type and Lager type) were used to carry out 0.5-liter fermentations (3.5 kg of 2-row pale malt, 30 g of cascade hops boiled 60 minutes with a specific gravity of around 1.035) in duplicates, they were incubated at their optimal fermentation temperatures (15 ± 2 °C or 25 ± 2 °C) for 17 days. The respective pitching rate was calculated and provided (0.75 million cells/mL/°P) in all cases. To calculate an estimation of the yeasts' ethanol production the original and final specific gravity of all beer wort was measured using a hydrometer (alla france, Chemillé, France) and to monitor sugar consumption, a measurement of the initial and final °Bx in beer wort was also taken. The decrease in specific gravity was correlated with the percentage of alcohol by volume present (ABV) by means of the following standard formula:

((1.05 x (Original gravity – Final gravity)) / Final gravity) / 0.79 X 100 = % ABV. Where 1.05 is grams of ethanol produced for every gram of CO_2 produced, and 0.79 is the density of ethanol. The apparent attenuation of the wort was also be calculated using the following formula:

AA = (OG - FG) / OG (Gravities are in specific gravity units)

RESULTS

Wild yeast strain isolation and screening

The results of this study's sampling assay show that wild yeasts can be found in sources like flowers, fruits, and ambient air. As is the case of several of the studies referenced, it was assumed that the isolates are wild yeasts given the locations from where they were obtained. From all the collected samples, 63 wild yeasts isolates were obtained.

Simple parametric statistics were used to analyze the data from the hedonic scales. This is consistent with the fact that the scales are described as ordinal in nature **(Cliff *et al.*, 2016)**. Normality tests to confirm non-normality of the data were performed (data not shown).

Attributes generated in flash profile analysis (Table 1) are consistent with those mentioned in literature. Some unpleasant sensory characteristics are the presence of acetaldehyde (poor wort fermentation), relatively high production of sulfur and phenolic compounds, as well as overproduction of esters **(Gibson, 2011)**. The presence of these compounds in some of the samples may have earned them a low hedonic score. Whereas attributes considered as positive are pleasant aroma and mouthfeel, as well as characteristic flavors like alcohol and fruitiness (esters) **(Verstrepen *et al.*, 2003)**.

Table 1 Attributes generated by panelists in flash profile analysis for all isolates throughout the 3 tastings. Aroma and in-mouth flavor are presented as acronyms A and F

Attributes	Frequency of mention
Sweet	18
F_Estery	16
F_Cooked Vegetable	10
F_Phenolic	10
F_Solvent	10
Astringent	8
Alcohol	8
A_Floral	8
F_Acetaldehyde	5
F_Sour	5
F_Diacetyl	4
F_Butyric	2
F_Earthy	2
A_Butyric	1
A_Diacetyl	1
A_Almond	1
A_Peach	1
Bitter	1

The factor analysis plot (Figure 1) shows that out of the 10 wild isolates with the highest hedonic scores (a mean liking of 7 or higher), there were 5 of them highly correlated to each other and the references (to a less extent to the lager reference), forming a group of samples at the upper right side of the plot in proximity to the attributes ester, solvent, alcohol, astringent and floral, which means that those attributes described them. The rest of the wild isolates formed a group in contiguity to the attributes sweet and cooked vegetable, except for one that was better described by phenolic.

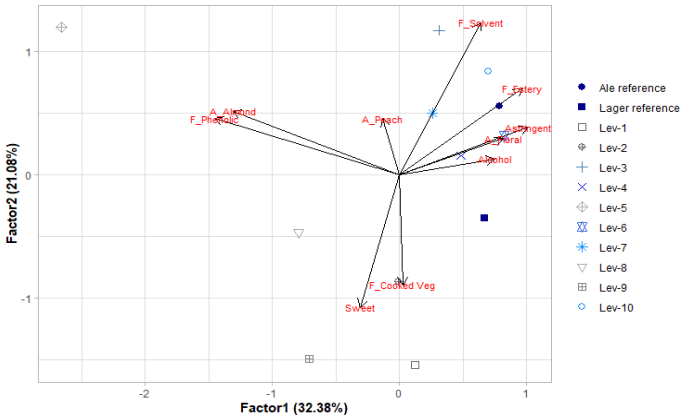

Figure 1 Factor analysis scores and loadings plot of flash profile sensory analysis of the 10 wild isolates with the highest hedonic scores and the reference yeasts

After the screening process, five yeasts of interest were selected (Table 2) based on their pleasant sensory characteristics (similarity in attributes to the references), their ability to maintain such characteristics without producing any detectable off flavors through all the fermentation cycles and their high hedonic rating.

Table 2 Initial and final °Bx and density values of wort from the ethanol yield estimation assay and estimated ABV and attenuation.

Isolate	Source	Fermentation temperature	Original Specific Gravity	Final Specific Gravity	ABV (%)	Attenuation (%)
Ale type reference	Commercial	25 °C	1.036	1.004	4.23	88.88
Lager type reference	Commercial	1 5°C	1.036	1.004	4.23	88.88
Lev-3	Wild berries surface	25 °C	1.034	1.010	3.16	70.59
Lev-4	Orange tree flowers	15 °C	1.034	1.010	3.16	70.59
Lev-6	Orange orchard ambient air	15 °C	1.034	1.009	3.23	72.06
Lev-7	Apple fermentation	15 °C	1.034	1.008	3.36	75.00
Lev-10	Apple fermentation	15 °C	1.034	1.006	3.70	82.35

Identification of yeast isolates of interest

The selected yeast strains (Lev-3, 4, 6, 7 and 10) were in all five cases identified as *Saccharomyces cerevisiae* according to the amplification of *MEX67* (Figure 2) as well as by restriction of the ITS with *HaeIII* (results not shown). None of the other primers specific for *S. paradoxus*, *S. mikatae, S. kudriavzevii, S. bayanus*, *S. arboriculus* showed PCR amplification. The sequences of the ITS-5.8S from the amplification of Lev-3 (Genbank MN525555), Lev-4 (Genbank MN525556), Lev-7 (Genbank MN525557 and Lev-10 (Genbank MN525558) all had more than 96% identity with *S. cerevisiae* (Genbank CP006466.1).

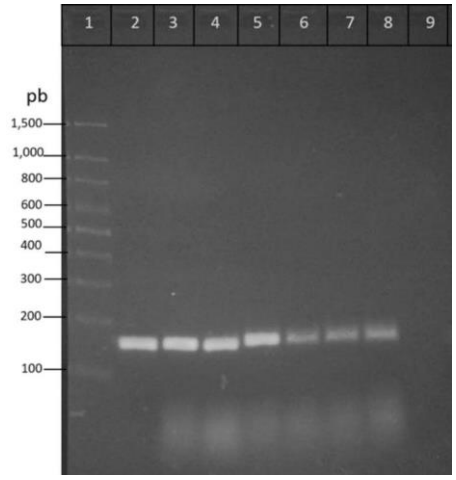

Figure 2 1.5% Agarose gel electrophoresis for MEX67 amplification (150 bp). In lane 1: 100 bp Ladder; Lane 2: *S. cerevisiae* S288c; Lane 3: SafAle S-04; Lane 4: Lev-3; Lane 5: Lev-4; Lane 6: Lev-6; Lane 7: Lev-7; Lane 8: Lev-10 and Lane 9: Negative control.

Growth curve and flocculation potential assay

When comparing the selected isolates to the Lager type reference it was observed that all wild yeasts showed comparable absorbance values at their maximum growth point (Figure 3A). In all cases, the selected isolates reached their maximum growth point before the reference yeast.

The isolated yeasts showed comparable values of absorbance to the reference (Figure 3B). It is worth noting that we used the lager (Saflager ™ S-189 Fermentis) yeast as a reference for growth and flocculation potential because all selected wild yeasts except for Lev-3, were incubated 15°C (Table 2).

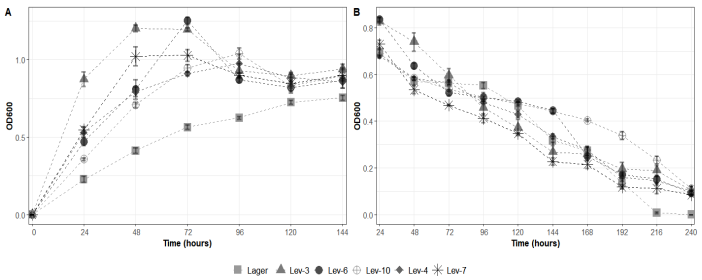

Figure 3 Optical density (A) and flocculation potential assay results (B) of selected isolates and Lager type reference strain

High gravity and ethanol tolerance

In this study's growth kinetics assay, the selected isolates reached an OD600 equivalent to 10^7 cells/mL (Figure 3A). This value was also observed in the control condition of this assay for all yeasts, including the references. At the 12°P of added glucose condition of this assay (concentration of sugars equivalent to a standard gravity industrial fermentation), all yeasts were able to maintain the expected number of viable cells ($1x10^7$ cells/mL) while Lev-3, Lev-4 and Lev-7 thrived in this condition reaching up to $1x10^8$ viable cells/mL. At 17°P (high gravity) and 25°P (very high gravity), it was observed that the reference strains were inhibited to varying degrees, in all cases the wild isolates exhibited higher cell density, with Lev-4 performing the best in very high gravity wort (Figure 4A). Sugar consumption was monitored by measuring °Bx over time, at 9 days (T2) of the very high gravity wort fermentation there was a high amount of sugar left (Figure 4B), so we kept measuring for 25 days.

At up to the 8% of added ethanol condition of this assay, the selected isolates and reference yeasts' growth was not affected. While at 12% of added ethanol it was observed that the reference strains were completely inhibited, Lev-3, Lev-6 and Lev-7 presented the greatest ethanol stress tolerance being able to reach and maintain the expected viable cell number ($1x10^7$ cells/mL) (Figure 5). At 16%, all yeasts except for Lev-7 were completely inhibited.

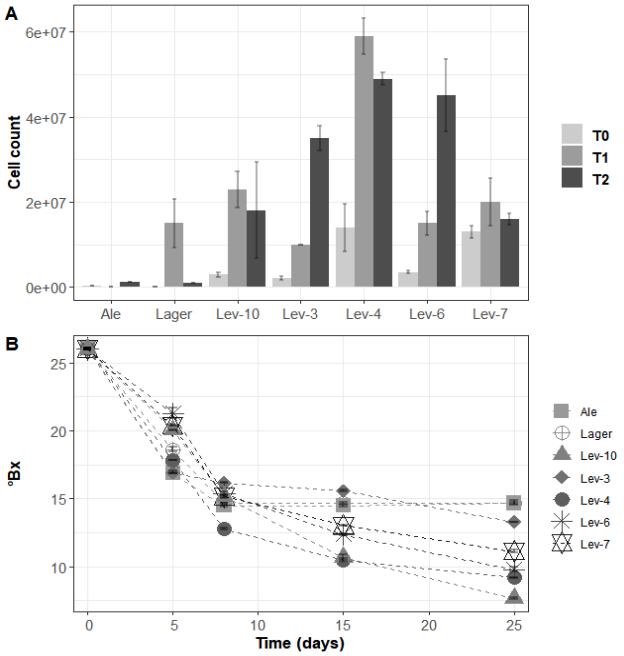

Figure 4 Cell number over time of selected isolates and the Lager and Ale type reference strains in the 25°P (added glucose) condition, T0: 1 day after inoculation; T1: 4 days after inoculation; T2: 9 days after inoculation (A). Brix degrees decline over time (B).

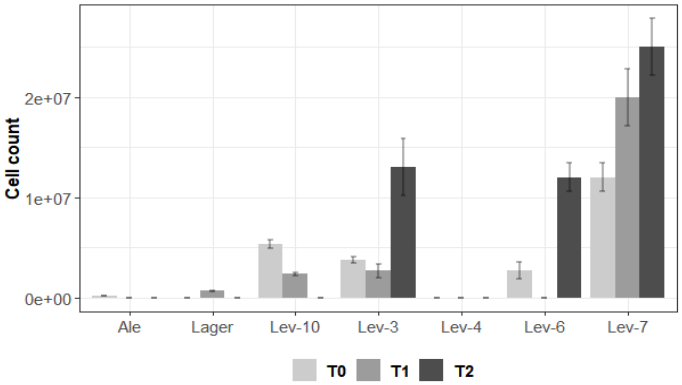

Figure 5 Cell number growth over time of selected isolates and the Lager and Ale type reference strains in the 12% added ethanol condition, T0: 1 day after inoculation; T1: 4 days after inoculation; T2: 9 days after inoculation.

Ethanol yield estimation

Reference yeasts (Lager type and Ale type) showed a very similar performance compared to each other regarding their ethanol production capacity and wort attenuation, which was higher than that of the selected wild yeasts in all cases. All selected yeasts showed similar attenuation values except for Lev-10 which exhibited a performance closer to that of the reference yeasts (Table 2).

DISCUSSION

We obtained 63 isolates from the samples gathered, of which 5 were selected based on their sensory characteristics (Table 2). The presence of wild yeasts found in the samples collected in this study corresponds to that reported in previous studies where diverse geographical locations were sampled. In a study performed in two widely separated areas: the Iberian Peninsula (Spain) and the Yucatan Peninsula (Mexico), the presence of wild yeasts in flower nectar was evaluated, finding high yeast cell population densities in the species sample **(Herrera *et al.*, 2009; Cliff *et al.*, 2016)**. In another study, osmotolerant wild

yeasts were successfully isolated from a total of 90 randomly collected samples of orchard soil, apple, and apple leaves from 10 apple orchards located in the Shaanxi province in China **(Wang *et al.*, 2015)**. Wild yeasts were also isolated from fruits such as grapes, molasses and cashew apples in a study conducted in India **(Tikka *et al.*, 2013)**. The screening process of the wild isolates in this study was based on the yeasts' ability to produce desirable sensory compounds, as well as to maintain those sensory characteristics throughout all the fermentation cycles. The latter aspect was also relevant because, normally, industrial scale fermentations are carried out with recycled yeast from previous fermentations, and this recycled yeast differs physiologically from the freshly propagated yeast from the pure culture **(Huuskonen *at al.*, 2010)**.

The nature and magnitude of yeast populations can vary widely and depend on the inherent composition of their habitat. There are surprising metabolic differences (rate of fermentation and flavor production) even among yeast species that are explained by inter-individual genetic variability **(Breunig *et al.*, 2014; Michel *et al.*, 2016; Cubillos *et al.*, 2019)**. Therefore, the varying sensory characteristics of the wild isolates of interest in this study, despite them being the same species, may be explained mainly by two reasons: the specific location and sample from which the yeast was isolated and the strain's metabolic characteristics (which depend to some extent on the former). *Saccharomyces cerevisiae* is predominantly found in association with human activities, but it can be frequently isolated from sugary foods and other sources unrelated to alcoholic beverage production **(Fay and Benavides, 2005)**, non-*Saccharomyces* wild strains in the total of isolates in this study may have been excluded because of the screening process.

When comparing growth of the selected isolates to the reference, all wild yeasts showed comparable absorbance values at their maximum growth point and in all cases, the selected isolates reached their maximum growth point before the reference yeast. The drop in OD600 at 96 hours in the growth curve (Figure 3A) may be explained by floc formation and thus, not all yeasts cells being resuspended, even after homogenization **(Stewart, 2018)**. The flocculation potential assay served as an indicator of the number of cells in suspension in the cultures (Figure 3B), which is an important characteristic in industrial yeast strains. Since the selected wild yeasts presented a similar behavior to a commercial lager strain, they may be appropriate for industrial fermentations **(Stewart, 2018)**.

As shown in the results, the selected isolates and reference yeast reached a similar number of cells while growing under normal conditions (Figure 3A). However, the high gravity assay showed that Lev-4 performed the best in very high gravity wort, while Lev-3, Lev-4 and Lev-7 thrived under normal conditions (Figure 4A). After 25 days of fermentation, the beer fermented by Lev-10 had the least number of sugars left (Figure 4B), and although 25 days is not a normal fermentation time for beers, with further studies this yeast could be used for other fermented beverages such as fruit wines.

These results agree with the inherent characteristics in *S. cerevisiae* strains, as there is large evidence of its efficient glucose utilization and high stress tolerance in general **(Stanley *et al.* 2010; Kayikci and Nielsen, 2015)**. Osmotolerant wild yeasts have also been successfully isolated in other similar studies obtaining diverse results in osmotic tolerance and species variability, **Wang *et al.* (2015)** used a glucose-enriched malt culture medium with different concentrations: 0% (control), 2%, 20%, 40% and 50% (p/v) to screen wild yeasts isolated from apple orchards for osmotic stress tolerance. Thirteen isolates were obtained and identified by the amplification of the ITS1-5.8S-ITS2 regions, the most common species were *Kluyveromyces marxianus*, *Hanseniaspora uvarum*, *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Candida tropicalis* and *Pichia kudriavzevii*. In another study conducted by **Suranska *et al.* (2016)** a YPD medium with 40% (w/v) and 50% (w/v) glucose was employed to screen wild isolates from vineyard grapes and spontaneously fermented musts for eonological properties. Four of the isolates, all of them identified as *Saccharomyces cerevisiae* by amplification of 5.8S rDNA gene regions, exhibited higher growth rate than a commercial *S. cerevisiae* reference strain (BS6) in these high sugar concentration conditions.

Sugar tolerance is an aspect with substantial variance among wild yeasts **(Ok and Hashinaga, 1997)**, besides the influence of the natural habitat and fermentation conditions on yeasts' osmotic stress tolerance, this trait is linked to the genetic factor, there is substantial evidence that the MAPK HOG (high osmolarity glycerol) pathway participates in the response to high sugar concentrations allowing *S. cerevisiae* cells to perceive and respond rapidly to altered osmolarity **(Gomar-Alba *et al.*, 2015)**.

The ethanol tolerance threshold observed in our assay is consistent with other studies where ethanol tolerant strains were obtained from similar samples. In a study conducted by **Arekar and Lele (2015)**, an ethanol tolerance of up to 12% with a radical biomass decrease observed thereafter was also observed. YPD broth containing varying concentrations of added ethanol from 6 to 15% (v/v) was used to screen wild yeasts isolated from tropical fruits. Two of the isolates identified as Saccharomyces cerevisiae showed similar ethanol tolerance to a *S. cerevisiae* reference strain (NCIM 3215). In another study carried out by **Tikka *et al.* (2013)** YPD medium with different concentrations of added ethanol: 6%, 7%, 8%, 9%, 10%, 11%, 12%, 12.5% 13%, 13.5%, 14% and 14.5% was used to screen wild yeasts isolated from fruits for ethanol stress tolerance. It was found through RAPD analysis that 7 out of 15 wild isolates obtained were *S.cerevisiae*. The most tested strains exhibited tolerance in a range of 7 to 12% of added ethanol with a very low tolerance rate afterwards. Some important effects of ethanol toxicity on yeasts are growth inhibition, reduced cell viability and enzyme inactivation. Cell membranes appear to be the main sites of ethanol damage; numerous studies conducted on *S.cerevisiae* have identified the composition of the plasma membrane essential for ethanol tolerance. The composition of the brewer's yeast membrane is influenced by the wort's composition and the fermentation temperature, which in turn are influenced by gene expression to allow adaptation to the environment **(Gibson, 2011)**.

A promising approach has been suggested by **Gibson *et al.* (2007)** to possibly improve brewing yeast performance in high gravity wort by supplementing with specific metal ions, lipids, and lipid components such as fatty acids and sterols, among others. This strategy may counter the toxic effects of these harsh brewing conditions on enzyme activity and the yeast cell membrane.

The attenuation and ethanol production of the isolated yeasts was like the reference strains (Table 2). Although usually domesticated strains show a more vigorous maltose fermentation than non-industrial yeast strains (thus, higher attenuation), there are cases where wild isolates may outperform commercial strains in maltose utilization as a carbon source **(Bell *et al.*, 2001; Lee *et al.*, 2011)**. Lower attenuation in some beer fermentations may be explained by the yeasts' glucose repression pathway (gene expression changes triggered by the presence of glucose), which has an impact in sugar uptake and produces sluggish fermentations and decreased yeast vitality **(Piddocke *et al.*, 2009; Kayikci and Nielsen, 2013; Lei *et al.*, 2016)**. In some cases, supplementing beer wort with maltose and malt triose has been shown to increase wort fermentability, even in high gravity brewing **(Herrera *et al.*, 2009; Piddocke *et al.*, 2009)**. It could be fruitful to perform additional high gravity assays supplementing with different sugars that could increase this study's isolates' attenuation percentage.

CONCLUSION

The data discussed in the present study demonstrates the wide possibilities of finding wild *Saccharomyces cerevisiae* strains in rich biodiverse environments that could provide new less conventional flavors to the beer industry and better fermentation performance; taking advantage of the rich biodiversity in microbial organisms that can be found in different parts of the world. The wild yeast isolates evaluated in this study were able to produce beer with desirable sensory attributes and exhibited characteristics that might be favorable for the brewing process such as vigorous growth and varying tolerance to high glucose and ethanol concentrations in comparison to the commercial reference strains. More specifically, Lev-4 and Lev-6, performed well in high gravity wort; Lev-7 had a high tolerance for ethanol and Lev-10 yielded similar ethanol concentrations to the commercial brewing yeasts and produced the beer with less residual sugars from a high gravity worth at 25 days of fermentation.

Most of the tests cited in this study, as well as those performed here, were carried out on a laboratory scale; hence with further studies on a larger scale these new yeasts could be adapted to use in commercial production of beer or other fermented beverages.

Acknowledgements

The present investigation has not received specific funding from public, commercial, or nonprofit organizations.

Declaration of interest

The authors declare that there are no conflicts of interest.

REFERENCES

Arekar C, Lele SS. (2015). Isolation and characterization of yeast from anthocyanin rich tropical fruits as wine starter culture. J Microbiol Biotechnol Food Sci, 5(2):146-154. https://doi.org/10.15414/JMBFS.2015.5.2.147-154

Bell P, Higgins V, Att P. (2001). Comparison of fermentative capacities of industrial baking and wild-type yeasts of the species Saccharomyces cerevisiae in different sugar media. Lett Appl Microbiol, 32: 224-229. https://doi.org/10.1046/j.1472-765X.2001.00894.x

Bokulich N ,Bamforth C. (2013). The Microbiology of Malting and Brewing. Microbiol Mol Biol Rev, 77(2): 157-172. https://doi.org/10.1128/MMBR.00060-12

Breunig J, Hackett S, Rabinowitz J, Kruglyak L. (2014). Genetic Basis of Metabolome Variation in Yeast. PLoS Geneti, 10(3):e1004142. https://doi.org/10.1371/journal.pgen.1004142

Campbell I, Priest F. (2003). Brewing microbiology: wild yeasts in brewing and distilling. Kluwer Academic/Plenum Publishers, New York.

Capece A, Romaniello R, Siesto G, Romano P. (2018). Conventional and Non-Conventional Yeasts in Beer Production. Fermentation, 4(2):38 https://doi.org/10.3390/fermentation4020038

Cliff M, Bejaei M, King M, Mcarthur D. (2016). Influence of Wine Education on Wine Hedonic and Confidence Ratings by Millennial Wine Consumers of Different Ethnicities. Beverages, 2(4):32. https://doi.org/10.3390/beverages2040032

Cubillos F, Krogerus K, Gibson B, Nikulin J, Grijalva-Vallejos N. (2019). Bioprospecting for brewers: Exploiting natural diversity for naturally diverse beers. Yeast, 36: 383-398. https://doi.org/10.1002/yea.3380

Dairou V, Siefferman J. (2002). A comparison of 14 jams characterized by conventional profile and a quick original method, the flash profile. J Food Sci, 67: 826-834. https://doi.org/10.1111/j.1365-2621.2002.tb10685.x

De Keersmaecker J. (1996). The mystery of lambic beer. Scientific American, 275:74-80. https://doi.org/10.1038/scientificamerican0896-74

Eeom YJ, Son SY, Jung DH, Hur MS, Kim CM, Park SY, Shin WC, Lee SJ, Auh JH, Kim GW, Park CS. (2018). Diversity analysis of Saccharomyces cerevisiae isolated from natural sources by multilocus sequence typing (MLST). Food Sci Biotechnol, 27:1119-1127. https://doi.org/10.1007/s10068-018-0335-z

Erten H, Tanguler H, Cak H. (2007). The effect of pitching rate on fermentation and flavour compounds in high gravity brewing. J Inst Brew, 113: 75-79. https://doi.org/10.1002/j.2050-0416.2007.tb00259.x

Fay J, Benavides J. (2005). Evidence for Domesticated and Wild Populations of Saccharomyces cerevisiae. PLoS Genetic, 1:0066-0071. https://doi.org/10.1371/journal.pgen.0010005

Fix G. (1999). Principles of brewing science: a study of serious brewing issues. Brewers Publications, Colorado, USA.

Fix G, Fix L. (1997). An Analysis of Brewing Techniques. Brewers Publications.

Gardes M, Bruns TD. (1993). ITS primers with enhanced specificity for basidiomycetes–application to the identification of mycorrhizae and rusts. Molecular ecology, 2: 113-118. https://doi.org/10.1111/j.1365-294X.1993.tb00005.x

Gibson B. (2011). 125th anniversary review: improvement of higher gravity brewery fermentation via wort enrichment and supplementation. J Inst Brew, 117: 268-284. https://doi.org/10.1002/j.2050-0416.2011.tb00472.x

Gibson BR, Lawrence SJ, Leclaire JPR, Powell CD, Smart KA. (2007). Yeast responses to stresses associated with industrial brewery handling. FEMS Microbiol Rev, 31:535-569. https://doi.org/10.1111/j.1574-6976.2007.00076

Gomar-Alba M, Morcillo-Parra MA, Del Olmo M. (2015). Response of yeast cells to high glucose involves molecular and physiological differences when compared to other osmostress conditions. FEMS Yeast Res, 15:1–14. https://doi.org/10.1093/femsyr/fov039

Hebly M, Brickwedde A, Bolat I, Driessen M, de Hulster E, van den Broek M, Pronk J, Geertman JM, Daran JM, Daran-Lapujade P. (2015). S. cerevisiae × S. eubayanus interspecific hybrid, the best of both worlds and beyond. FEMS Yeast Res, 15:1-14. https://doi.org/10.1093/femsyr/fov005

Herrera CM, De Vega C, Canto A, Pozo MI. (2009). Yeasts in floral nectar: a quantitative survey. Ann Bot, 103(9): 1415–1423. https://doi.org/10.1093/aob/mcp026

Huuskonen A, Markkula T, Vidgren V, Lima L, Mulder L, Geurts W, Londesborough J. (2010). Selection from industrial lager yeast strains of variants with improved fermentation performance in very-high-gravity worts. Appl Environ Microbiol, 76(5): 1563-1573. https://doi.org/10.1128/AEM.03153-09

Kayikci O, Nielsen J. (2015). Glucose repression in Saccharomyces cerevisiae. FEMS Yeast Res, 15(6):1-8. https://doi.org/10.1093/femsyr/fov068

Kihn J, Masy C, Mestdagh M, Rouxhet P. (1988). Yeast flocculation: factors affecting the measurement of flocculence. Can J Microbiol, 34: 779-781. https://doi.org/10.1139/m88-132

Lee Y, JuChoi Y, Lee S, Park J, Shim J, Park K, Kim J. (2011). Screening wild yeast strains for alcohol fermentation from various fruits. Mycobiology, 39: 33-39. https://doi.org/10.4489/MYCO.2011.39.1.033

Lei H, Xu H, Feng L, Yu Z, Zhao H, Zhao M. (2016). Fermentation performance of lager yeast in high gravity beer fermentations with different sugar supplementations. J Biosci Bioeng, 122(5):583-588. https://doi.org/10.1016/j.jbiosc.2016.05.004

Lentz M, Putzke T, Hessler R, Luman E. (2014). Genetic and physiological characterization of yeast isolated from ripe fruit and analysis of fermentation and brewing potential. J Inst Brew, 120(4): 559-564. https://doi.org/10.1002/jib.154

Lim J. (2017). Hedonic scaling : A review of methods and theory Hedonic scaling : A review of methods and theory. Food Qual Prefer, 22: 733-747. https://doi.org/10.1016/j.foodqual.2011.05.008

McCullough MJ, Clemons KV, McCusker JH, Stevens DA. (1998). Intergenic transcribed spacer PCR ribotyping for differentiation of Saccharomyces species and interspecific hybrids. J. Clin. Microbiol, 36(4): 1035-103. https://doi.org/10.1128/JCM.36.4.1035-1038.1998

Michel M, Meier-Dörnberg T, Jacob F, Methner FJ, Wagner RS, Hutzler M. (2016). Review: pure non-Saccharomyces starter cultures for beer fermentation with a focus on secondary metabolites and practical applications. J Inst Brew, 122(4): 569-587. https://doi.org/10.1002/jib.381

Mosher R. (2009). Tasting Beer: An insider's guide to the world's greatest drink. 95-100. Storey Publishing, Massachusetts, USA.

Muir A, Harrison E, Wheals A. (2011). A multiplex set of species-specific primers for rapid identification of members of the genus Saccharomyces. FEMS Yeast Res, 11(7):552-56. https://doi.org/10.1111/j.1567-1364.2011.00745.x

Nedovic V, Gibson B., Mantzouridou TF, Bugarski B, Djordjevic V, Kalusevic A, Paraskevopoulou A, Sandell M, Smogrovicova D, and Yilmaztekin M. (2014). Aroma formation by immobilized yeast cells in fermentation processes, Yeast, 32(1):173-216. https://doi.org/10.1002/yea.3042

Ok T, Hashinaga F. (1997). Identification of sugar-tolerant yeasts isolated from high-sugar fermented vegetable extracts. J Gen Appl Microbiol,; 43:39-47. https://doi.org/10.2323/jgam.43.39

Olaniran AO, Hiralal L, Mokoena MP, Pillay B. (2017). Flavour-active volatile compounds in beer: production, regulation and control. J Inst Brew, 123(1): 13-23. https://doi.org/10.1002/jib.389

Osburn K, Caputo R, Miller J, Bochman M. (2017). Saccharomyces cerevisiae strain YH166: a novel wild yeast for the production of tropical fruit sensory attributes in fermented beverages. bioRxiv, 149732. https://doi.org/10.1101/149732

Osorio-Cadavid E, Ramírez-Castrillón M, López-Arboleda A, Mambuscay L. (2009). Standardization of a simple protocol for yeast genomic DNA extraction. Revista Colombiana de Biotecnología, 11:125-131.

Parapouli M, Vasileiadis A, Afendra AS, Hatziloukas E. (2020). Saccharomyces cerevisiae and its industrial applications. AIMS Microbiol, 6(1):1-31. https://doi.org/10.3934/microbiol.2020001

Piddocke MP, Kreisz S, Heldt-Hansen HP, Nielsen KF, Olsson L. (2009). Physiological characterization of brewer's yeast in high-gravity beer fermentations with glucose or maltose syrups as adjuncts. Appl Microbiol Biotechnol, 84:453-64. https://doi.org/10.1007/s00253-009-1930-y

R Core Team. (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Stanley D, Bandara A, Fraser S, Chambers P, Stanley G. (2010). The ethanol stress response and ethanol tolerance of Saccharomyces cerevisiae. J Appl Microbiol, 109(1):13-24. https://doi.org/10.1111/j.1365-2672.2009.04657.x

Steensels J, Snoek T, Meersman E, Nicolino MP, Voordeckers K, Verstrepen KJ. (2014). Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol Rev, 38(5): 947–995. https://doi.org/10.1111/1574-6976.12073

Stewart G. (2018). Yeast Flocculation – Sediment and Flotation. Fermentation, 4(2): 28. https://doi.org/10.3390/fermentation4020028

Suranska H, Vranova D, Omelkova J. (2016). Isolation, identification and characterization of regional indigenous Saccharomyces cerevisiae strains. Braz J Microbiol, 47(1):181-190. https://doi.org/10.1016/j.bjm.2015.11.010

Tang Y, Horikoshi M, Li W. (2016). ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages. The R Journal 8.2, 478-489.

Tikka C, Osuru HP, Atluri N, Raghavulu PCV, Yellapu NK, Mannur IS, Bhaskar M. (2013). Isolation and characterization of ethanol tolerant yeast strains. Bioinformation, 9(8): 421-425. https://doi.org/10.6026/97320630009421

Verstrepen KJ, Derdelinckx G, Dufour JP, Winderickx J, Thevelein JM, Pretorius IS, Delvaux FR. (2003). Flavour-active esters: adding fruitiness to beer. J Biosci Bioeng, 96(2): 110-118. https://doi.org/10.1016/S1389-1723(03)90112-5

Vidgren V, Londesborough J. (2011). 125th anniversary review: Yeast flocculation and sedimentation in brewing. J Inst Brew, 117: 475-487. https://doi.org/10.1002/j.2050-0416.2011.tb00495.x

Wang H, Hu Z, Long F, Niu C, Yuan Y, Yue T. (2015). Characterization of osmotolerant yeasts and yeast-like molds from apple orchards and apple juice processing plants in China and investigation of their spoilage potential. J Food Sci, 80(8): M1850–M1860. https://doi.org/10.1111/1750-3841.12946

Wickham H. (2016). ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag New York.