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Bioethanol production from sugarcane molasses by co-fermentation of *Saccharomyces cerevisiae* isolate TA2 and *Wickerhamomyces anomalus* isolate HCJ2F-19

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Abstract

Purpose Co-culturing is a widely used method to improve bioethanol production from biomass enriched in fermentable sugars. This study aims to produce bioethanol from sugarcane molasses by simultaneous co-fermentation of *S. cerevisiae* isolate TA2 and *W. anomalus* isolate HCJ2F-19.

Methods Response surface methodology (RSM) based on the central composite design (CCD) was employed to optimize fermentation conditions, including mixing rate (110–150 rpm), temperature (25–35 °C), molasses concentration (25–35 ^{o}brix), and incubation time (36–72 h). The ethanol concentration was analyzed using HPLC equipped with a UV detector.

Results The monoculture*S. cerevisiae* isolate TA2 produced 17.2 $g.L^{-1}$ of ethanol, 0.33 $g.g^{-1}$ of ethanol yield, and 0.36 $g.L^{-1}.h^{-1}$ of productivity compared to *W. anomalus* isolate HCJ2F that produced 14.5 $g.L^{-1}$, 0.30 $g.g^{-1}$ and 0.28 $g.L^{-1}.h^{-1}$ ethanol, ethanol yield, and productivity under laboratory conditions, respectively. In comparison to single cultures of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F, the co-fermentation using both isolates showed an increased ethanol yield of 29% and 53% compared to the single species fermentations, respectively. The results showed that the growth of *W. anomalus* HCJ2F-19 and *S. cerevisiae* TA2 was not influenced by each other during the co-fermentation process. The one variable at a time optimization (OVAT) analysis resulted in an ethanol concentration of 26.5 $g.L^{-1}$ with a specific yield and productivity of 0.46 $g.g^{-1}$, 0.55 $g.L^{-1}.h^{-1}$, respectively, at pH 5.5, 25 ^{o}brix, 48 h, 150 rpm, 30 °C, 60:40 inoculum ratio, and 10% overall inoculum size. The maximum ethanol concentration of 35.5 $g.L^{-1}$ was obtained by co-fermentation using the RSM-CCD tool at 30 ^{o}brix, 30 °C, 54 h, and 130 rpm.

Conclusion The results suggested that the co-fermentation of *S. cerevisiae* isolate TA2 and *W. anomalus* isolate HCJ2F improves bioethanol production from sugar cane molasses under optimum fermentation conditions.

Keywords Co-fermentation, Optimization, Bioethanol, Sugarcane molasses, Ethanol yield, Stress tolerance, Statistical optimization

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Introduction

Global warming, fossil fuel dependency, and food and energy security are among the primary challenges facing the modern world (Robak & Balcerek 2018). Fossil fuel is used as the main source of energy in the transportation industry, consuming an amount of 78.4% of the global total energy consumption (REN21, 2017). The growing global population and industrialization increased energy demand, yet conventional fossil fuel supplies are limited and are also debated because of environmental issues, mainly due to the generation of bulky greenhouse gas (GHG) emissions to the environment contributing to global warming (Heidari & Pearce 2016). Petroleum is the main fossil-derived energy source, accounting for 80% of the global energy market (Branco et al. 2018). Research interest in alternative biobased energy sources is growing because of the need to reduce the use of fossil fuels, unstable fossil fuel supplies, depleting fossil resources, rising energy demand, and emerging environmental concerns, such as pollution and GHG emissions (Demirbas 2009; Dhyani & Bhaskar 2018; Mohapatra et al. 2017). Because of these considerations, the production of sustainable and environmentally friendly alternative energy, such as biofuel from sustainable renewable sources, has received widespread attention (Azadi et al. 2017).

The use of biofuels represents an attractive renewable alternative to diminishing the dependence on fossil fuels, the production of GHG emissions, and the consumption of natural oil (Braz et al. 2019; Choudhary et al. 2017; Zhou et al. 2012). Among biofuels, bioethanol is the most dominant liquid biofuel and environment-friendly bioenergy source (Balat & Balat 2009; Demain et al. 2005; Dias et al. 2011; Hill et al. 2006). Several countries such as the USA, Brazil, China, Canada, India, Thailand, Argentina, and EU nations, have already taken steps towards increasing the production of bioethanol. The United States of America (USA) is the world's largest producer, accounting for 59% of bioethanol production followed by Brazil with 27% (REN21, 2017). In 2017, the addition of bioethanol to gasoline resulted in a reduction of transportation-related greenhouse gas emissions of 43.5 million metric tons of CO_2, equaling the removal of 9.3 million cars from the road.

Bioethanol can be produced from commonly available biomass containing fermentable free sugars, including lignocellulosic materials (e.g., wood, straw, and bagasse), starchy materials (e.g., wheat, corn, and barley), and sugar-based feedstocks (e.g., sugarcane, sugar beet, and molasses) (Liew et al. 2014; Mussatto et al. 2010; Nigam & Singh 2011).

1G bioethanol refers to ethanol produced from starchy feedstock (e.g., corn, maize, wheat, barley, cassava, potato) and sucrose feedstock (e.g., sugarcane, sugar beet, molasses, sweet sorghum) (Ho et al. 2014; Lennartsson et al. 2014). European countries primarily use sugar beets (e.g., France, Germany, UK, Czech Republic, Belgium, and Austria), wheat (Belgium, France, and UK), corn (e.g., Central Europe, the Netherlands, and Spain), and beet pulp (e.g., Austria and Belgium) as feedstocks for bioethanol production. In 2022, over 28,000 million gallons of bioethanol was produced worldwide. The United States and Brazil ranked the first and second bioethanol-producing countries using sugarcane juice and corn starch, respectively. The US produced almost 15, 361 million gallons of bioethanol, which constitutes 54% of the global output. Brazil produced approximately 7,400 million gallons of bioethanol covered 26% of the global bioethanol production. The member states of the European Union together produced 1,460 million gallons of bioethanol from various biomasses. The growing population and increased production of 1G bioethanol, however, have raised serious concerns about the sustainability of the practice over the long run. These concerns include the potential threat to global food and feed security, the socio-economic and environmental effects of large-scale production, and soil contamination from distillation residues (Buijs et al. 2013).

Second-generation (2G) bioethanol production is attractive since it uses low-cost waste resources that do not compete with human food or animal feedstocks (Domínguez-Bocanegra et al. 2015; Thompson & Meyer 2013). However, industrial scale bioethanol production still faces obstacles such as suitable feedstock acquisition, production costs, the technology needed for production and pretreatments, and environmental problems that need to be overcome to use the 2G feedstocks effectively (Bellido et al. 2013; Mathew et al. 2014). Several alternatives for bioethanol production technologies from agro-industrial wastes are being investigated, such as separate hydrolysis and fermentation (SHF); separate hydrolysis and co-fermentation (SHCF); simultaneous saccharification and fermentation (SSF); simultaneous saccharification and co-fermentation (SSCF); pre-saccharification followed by simultaneous saccharification and fermentation (PSSF), and consolidated bioprocessing (CBP) (Carrillo-Nieves et al. 2019). 2G bioethanol is produced from agricultural and industrial food waste, market and household food waste, and horticultural waste that all contain fermentable carbohydrates that are converted to ethanol (Vučurović et al. 2012). The production of bioethanol from agro-industrial wastes is important from environmental and food production points of view (Vučurović et al. 2012).

Sugarcane molasses is a first-generation (1G) feedstock that is extensively used to produce valuable products, such as bioethanol, and other chemicals, such as adipic

acid (Cronjé et al. 2023; Moonsamy et al. 2022). Currently, 1G biomasses produce 99% of the global ethanol yield and its production processes are well-established (Moonsamy et al. 2022). Sugarcane juice and sugarcane molasses have been an important source of ethanol production in Brazil, India, and South Africa (Farzad et al. 2017). Ethiopia produces 542,316 tons sugar cane molasses annually, which is used by both state and private bioethanol companies to make bioethanol (Gebreegziabher et al. 2017). Sugarcane molasses is a thick and concentrated sugar-containing liquid biomass produced at the first, second, and third stages of the crystallization process in the sugar mill (Moonsamy et al. 2022). Molasses contains 60% sucrose that are hydrolyzed into glucose and fructose during the fermentation process (Dodić et al. 2009). In addition, sugarcane molasses also contains minerals, such as calcium 150–2000 mg L^{-1}, potassium 300–12,000 mg L^{-1}, and magnesium 80–3900 mg L^{-1} (Basso et al. 2011), but the high concentrations of these minerals inhibit the enzyme activity, specifically of invertase that converts sucrose into reducing sugar (Chotineeranat et al. 2010). Decalcification with H_2SO_4 is pretreatment method that can lower the mineral levels in sugarcane molasses, particularly calcium (Jayanti et al. 2019).

Microorganisms are employed for the conversion of biomass into bioethanol. Bacteria, such as *Zymomonas mobilis* and genetically modified *Escherichia coli*, can convert fermentable sugars (i.e., glucose, sucrose, and fructose) to bioethanol (Aditiya et al. 2016). The yeast *Saccharomyces cerevisiae*, however, is the most widely used microorganism for bioethanol production, and has a tolerance for low pH values, high temperatures, ethanol, and certain inhibitors, as well as the ability to use a variety of disaccharides (such as sucrose and maltose) and hexoses (such as glucose, mannose, and galactose) (Balat 2011). In addition, *S. cerevisiae* is insensitive to bacteriophage infection which is relevant to the industrial-scale fermentation process. The fermentative characteristics of *S. cerevisiae* are mostly impaired as the ethanol concentration increases during ethanol formation. Ethanol can become toxic to the yeast cells leading to decreased viability, inducing stress responses, and triggering changes in gene expression and metabolic pathways. The elevated ethanol levels can also inhibit the activity of enzymes involved in various metabolic pathways that can affect the overall fermentation process and may lead to the incomplete conversion of sugars to ethanol. This is a significant drawback of the use of *S. cerevisiae* in industrial settings where maximizing ethanol production from e.g. sugarcane molasses is a primary goal. Moreover, *S. cerevisiae* is unable to ferment pentose sugars present in lignocellulosic substrates, i.e., xylose, because it lacks the metabolic mechanism (i.e., the oxidoreduction pathway) required to convert xylose into xylulose, which would be incorporated into the pentose phosphate pathway (PPP) (Song et al. 2019). Combining non-conventional pentose fermenting yeast species with *S. cerevisiae* in biofuel fermentation is a possible way to improve bioethanol production from xylose-containing biomasses (Wu et al. 2023).

Nowadays, four strategies are being used for the efficient production of bioethanol from biomasses containing fermentable sugars. The first strategy uses single-culture fermentation with a single microorganism, usually a specific strain of yeast, bacteria, or a filamentous fungal species, to convert sugars into ethanol. *S. cerevisiae* is one of the most commonly used microorganisms for bioethanol production in single-culture fermentations due to its high ethanol yield and efficiency in converting hexose sugars to ethanol (Kasavi et al. 2012; Lin et al. 2012). The second strategy involves co-fermenting *S. cerevisiae* with a strain of a non-conventional yeast species, which tolerates a range of stress conditions and that can e.g. ferment C-5 sugars, thus allowing efficient sugar conversion from biomass or hydrolysate, (Singh et al. 2014). Efficient co-fermentation of ethanol in a single procedure reduces capital expenditures and contamination risks (Wu et al. 2023). With this method, non-competitiveness and synergistic characteristics of the yeast species and—strains are required. However, it is challenging to provide the specific conditions needed for the co-fermentation of the sugars by both conventional and non-conventional yeast species (Lin et al. 2016; Morales et al. 2015). The third strategy is mostly applied in the case of lignocellulosic biomasses (or so-called 2G biomasses). Utilization of genetically engineered xylose-fermenting *S. cerevisiae* strains with heterologous xylose metabolic pathways is a highly efficient method for this purpose (Van Maris et al. 2007). With this strategy, the efficiency of the yeast for xylose conversion into ethanol remains challenging because of xylitol production during the ethanol fermentation process resulting in lower ethanol yields that still need to be overcome (Moysés et al. 2016). The fourth strategy is the utilization of a non-conventional xylose-fermenting yeast species, such as *Scheffersomyces* (also known as (aka)) *Pichia stipitis, Scheffersomyces* (aka *Candida*) *shehatae*, and *Pachysolen tannophilus.* Yücel and Aksu (2015) studied the yeast *Scheffersomyces stipitis* (cited as *Pichia stipitis*) for the fermentation of sugar beet pulp hydrolysates to produce a high ethanol yield. *S. stipitis*, however, has a lower tolerance for ethanol and sugar concentration than *Saccharomyces* strains, which restricts its use for large-scale ethanol fermentation (Shi et al. 2014). Apart from the aforementioned strategies, it is important to optimize the critical fermentation

variables, such as inoculum size, sugar content, agitation rate, incubation temperature, pH, and fermentation time, since these influence the bioethanol yield during the fermentation process (El-Mekkawi et al. 2019).

Wickerhamomyces anomalus is a non-conventional yeast species that was previously classified as *Pichia anomala, Hansenula anomala,* and *Candida pelliculosa.* The species was placed into the genus *Wickerhamomyces* after a phylogenetic analysis of gene sequences (Kurtzman et al. 2008). *W. anomalus* is used for the production of alcohol and is mainly used to make wine in which it also enhances the complexity of wine aromas (Ciani & Comitini 2015; Satora et al. 2014). *W. anomalus* is a less stress-tolerant yeast that can produce a comparable bioethanol yield from a variety of biomass hydrolysates if compared to *Saccharomyces* species, but with a longer fermentation duration (Ruyters et al. 2015; Zha et al. 2013). The yeast strains used in the present work were previously isolated from sugarcane molasses from the Fincha sugar factory, in Fincha, Ethiopia (Hawaz et al. 2022). The isolate *S. cerevisiae* TA2 demonstrated stress tolerance characteristics, including tolerances to high amounts of ethanol (i.e., 18% ($v.v^{-1}$)), high glucose concentrations (i.e., 50% ($w.v^{-1}$), as well as good fermentative characteristics (Hawaz et al. 2022). On the other hand, *W. anomalus* isolate HCJ2F-19 showed a lower ethanol tolerance (i.e., 10% (v/v)) with good fermentative characteristics. In this study, we used a co-fermentation technique to investigate the feasibility whether it is possible to increase ethanol production from sugarcane molasses, by co-fermenting two lower ethanol-producing yeasts, i.e., *S. cerevisiae* strain TA2 and *W. anomalus* strain HCJ2F, as reported by Hawaz et al. (2022). In addition, the co-fermentation dynamics and ethanologenic capabilities of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 were evaluated. The ethanol fermentation parameters were optimized using the design of experts (DoE) tool. To the best of our knowledge, this is the first study to demonstrate a higher bioethanol yield obtained by co-fermentation of two lower ethanol-producing yeast strains using sugarcane molasses as a primary substrate. For this purpose, a thorough investigation and analysis of propagation and fermentation dynamics of the monocultures and co-culture was carried out.

Materials and methods

Feedstock collection and pretreatment

Fresh concentrated sugarcane molasses of approximately 63°brix was obtained using sterile plastic containers from Fincha Sugar Factory, Wollega province in West-Central Ethiopia (8° 31′ N 39° 12′ E), an area with a humid subtropical climate with average annual temperatures of 31 °C. Before collection, an automated agitator was used to homogenize the samples that were collected during the dry season in March and April 2020. The molasses was immediately transported in an icebox to the Fermentation Laboratory, Department of Biotechnology, Addis Ababa Science and Technology University. After arrival at the laboratory, the sample was kept at room temperature (25 ± 2 °C) in airtight containers prior to its usage. The molasses was diluted with distilled water in an autoclavable bottle according to the desired concentration set by the experiment. For yeast cell propagation, the molasses concentration was adjusted to 8, 10, and 12 °brix, whereas for ethanol fermentation it was diluted to 30 °brix. The physicochemical characteristics of the sugarcane molasses were provided by the sugar factory and contained total solid content of 75% ($w.v^{-1}$), organic solid content of 66% ($w.v^{-1}$), an inorganic solid content of 12% ($w.v^{-1}$), and total sugar content of 54% ($w.v^{-1}$) (i.e., sucrose 32% ($w.v^{-1}$), fructose 10% ($w.v^{-1}$), and glucose 8% ($w.v^{-1}$).

Furthermore, the molasses was pretreated with concentrated 99.8% H_2SO_4 until the pH of the solution reached 4.6. The mixture was then heated at 90 °C for 2 h to remove unwanted constituents and left overnight at room temperature (De Vasconcelos et al. 2004; Malik 2016; Rahman et al. 2013). An amount of 2 $g.L^{-1}$ di-ammonium phosphate (DAP) was added to the solution and a magnetic stirrer was used to homogenize the mixture. Finally, using H_2SO_4, the pH was once more set at 4.6 before being autoclaved at 121 °C for 15 min. The autoclaved and treated solution was standing overnight under the safety cabinet to let undesirable constituents sediment, i.e., sludge, ash contents, and other particulates (Arshad et al. 2008). The clear upper suspension was used further in the experiments.

Yeast culture sources and inoculum preparation

The ethanol-tolerant yeast, *Saccharomyces cerevisiae* isolate TA2 (rDNA ncbi accession number OM367901), and a less ethanol-tolerant yeast, *Wickerhamomyces anomalus* isolate HCJ2F-19 (rDNA ncbi accession number OM367888) were obtained from the culture collection laboratory of the Department of Biotechnology, Addis Ababa Science and Technology University, Ethiopia. The stock cultures were preserved in 20% glycerol stock solution at -80 °C. Strains from long-term stocks were streaked on YPD agar that contains (in $w.v^{-1}$) 10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ dextrose, and 20 $g.L^{-1}$ agar (Naito et al. 2019) and incubated overnight (24 h) at 30 °C. For each strain, single colonies were used to inoculate 10 mL of YPD broth. Each yeast strain was aerobically incubated at 30 °C with vigorous shaking at 150 rpm for 24 h. The pH of the medium was adjusted with concentrated H_2SO_4 to 4.6. The yeast cells were

subsequently harvested by centrifuging at 5,000 g for 2 min at 4 °C. The pellet was re-suspended again using sterile distilled water and re-centrifuged to obtain a clear yeast cell pellet. Finally, a sterile phosphate buffer (pH 5.0) was used to re-suspend the pellet to obtain an optical density (OD) of 0.6 at 600 nm, which is equivalent to 1×10^6 cells.mL^{-1} for use as an inoculum for culture propagation using molasses (Boboye & Dayo-Owoyemi 2009; Raina et al. 2020).

Cultural characteristics of the yeast strains

The yeast isolates *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 were previously isolated from sugarcane molasses of Fincha Sugar factory in Ethiopia (Hawaz et al. 2022). These yeast strains were employed for bioethanol production, due to their capacity for diverse sugar fermentation and to withstand stress conditions (Hawaz et al. 2022). Both yeast strains can ferment sugars such as glucose, galactose, maltose, sucrose, trehalose, raffinose, and fructose. The isolate *S. cerevisiae* TA2 tolerates 18% ethanol, 45 °C, and a sugar content of 50%, but *W. anomalus* HCJ2F-19 tolerates is less ethanol (viz., 10% (v.v^{-1}) (Hawaz et al. 2022).

Propagation of yeast cultures using molasses

The yeast strains were propagated separately in Erlenmeyer flasks with different working volumes and substrate concentrations. Initially, 100 mL of 8 °brix molasses was used to propagate the yeast cells. Concentrated H_2SO_4 (99.8%) was used to adjust the propagation medium's pH to 4.6. Moreover, the medium was additionally supplemented with 4 g.L^{-1} di-ammonium phosphate (DAP) as a nitrogen source and autoclaved at 121 °C for 15 min (De Vasconcelos et al. 2004; Hawaz et al. 2022). The medium was thereafter allowed to cool overnight under a safety hood to let unwanted debris to sediment. Thereafter, the clear suspension was transferred aseptically under the safety hood to a sterilized Erlenmeyer flask of 250 mL. Finally, 0.1 mL of aliquots of active yeast cultures were inoculated to the molasses propagation medium (MPM) and vigorously shaken at 150 rpm at 30 °C for 24 h. Aliquots were drawn at 24 h and the propagation dynamics were determined by measuring the brix, pH, and cell viability using a refractometer, pH meter, and hemocytometer, respectively.

Yeast cultures that had been propagated under the same conditions were transferred to the second stage of propagation, which contained 10 °brix, and from the second stage to the third stage, which contained 12 °brix of molasses. The outlined protocol was followed for propagation, substrate treatment, supplementing, and measurement of the fermentation parameters. The propagated yeast cultures were used for the fermentation of molasses.

Mono-culture fermentation

The potential of each yeast strain to produce bioethanol was investigated for each isolate separately using a batch fermentation system. The fermentation procedure used the conditions obtained by Hawaz et al. (2022). The mono-culture fermentation process was performed using a 2 L Erlenmeyer flask with a working volume of 1 L. The inoculation size of 10% (v.v^{-1}) was added to 30 °brix of molasses fermentation medium supplemented with 2 g.L^{-1} of DAP. Before inoculation, the medium was sterilized at 121 °C for 15 min and the solution was left overnight under a safety hood. The upper clear syrup was poured into a new sterilized Erlenmeyer flask (2 L) inoculated with an overnight-growing yeast cell culture (Table 2) and incubated under anaerobic conditions created by an anaerobic chamber at 30 °C for 72 h at 150 rpm. Samples were drawn periodically after every 6 h and measurements of cell viability, alcohol percent, ethanol concentration, degree brix, pH, and reducing sugar (RS) were carried out using a hemocytometer, Ebulliometer, HPLC, refractometer, pH meter, and DNS method, respectively.

Co-culture fermentation

The co-fermentation of *S. cerevisiae* isolate TA2 and *W. anomalus* isolate HCJ2F-19 was carried out at pH 4.6, 30 °C, 30 °brix, 2 g.L^{-1} DAP supplement, mixing rate 150 rpm, incubation period 72 h (Hawaz et al. 2022), inoculum ratio of (20/80, in v.v^{-1}) and an overall inoculum size of 10% (v.v^{-1}) (Wu et al. 2023). The yeast *S. cerevisiae* TA2 (highly ethanol tolerant) was first inoculated with a volume of 200 mL (2.9×10^8 cells.mL^{-1}) (Fig. 1) into 200 mL molasses fermentation medium, and the culture was then incubated anaerobically at 30 °C and 150 rpm for 24 h. After a 24 h fermentation period, the relatively lower ethanol-tolerant yeast *W. anomalus* HCJ2F-19 suspension was added with a volume of 800 mL (3.5×10^8 cells.mL^{-1}) (Fig. 2) and 800 mL fermentation medium, and the mixed culture was further incubated for 72 h with continuous orbital shaking at 150 rpm and 30 °C. During 72 h, fermented samples were withdrawn aseptically for quantitative examination every 6 h. The fermentation was considered complete when the brix level of the molasses fermentation medium remained stable (Duarte et al. 2009). Fermentation parameters such as the degree brix, pH, RS, ethanol concentration, sugar consumption rate, and cell viability were analyzed as indicated above.

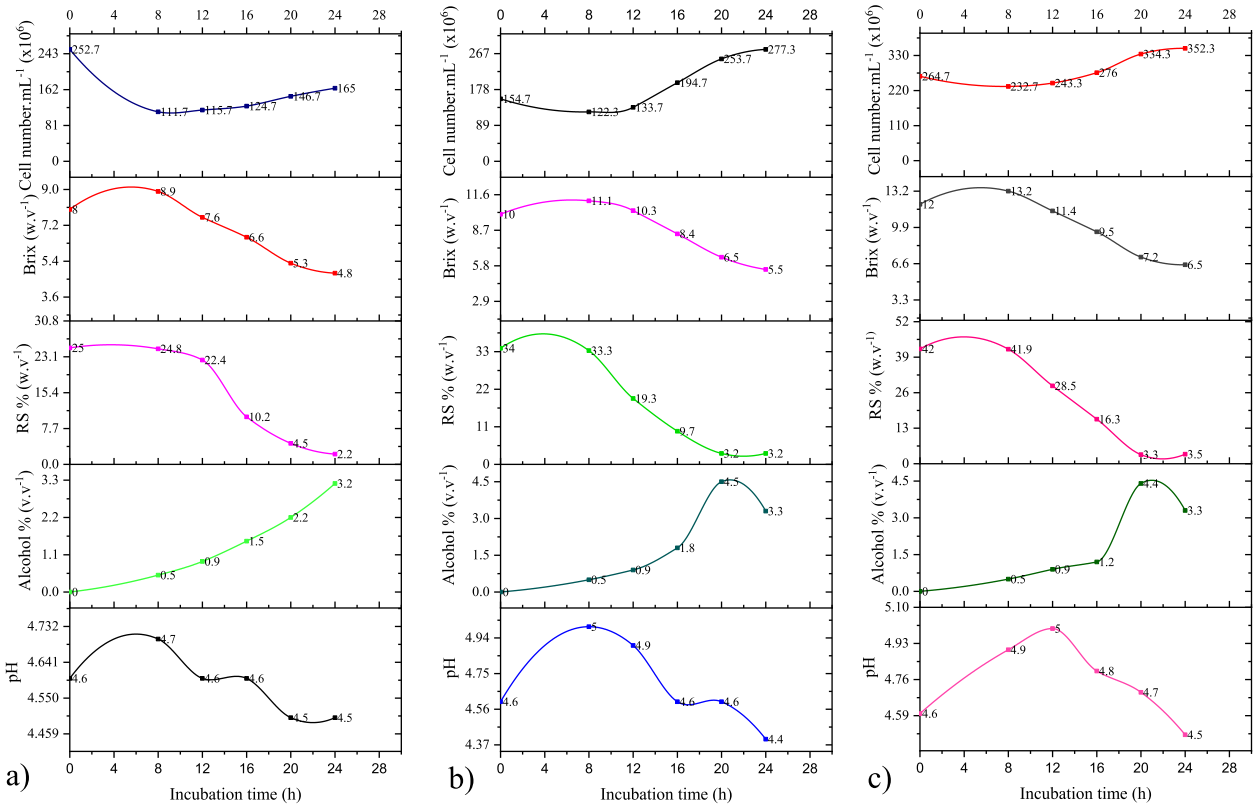

Fig. 1 Propagation dynamics of *W. anomalus* isolate HCJ2F-19 under (**a**) 8 °brix, (**b**) 10 °brix, and (**c**) 12 °brix at pH 4.6, temperature 30 °C, DAP concentration 2 g.L^{-1}, and molasses concentration 30 °brix (w.v^{-1}) (conditions taken from the literature). Under the third propagation phase (12 °brix), a viable yeast cell density of 3.5×10^8 cells.mL^{-1} was recorded at a final pH of 4.5 after 24 h of incubation

One variable at a time optimization

To maximize the alcohol fermentation performance of the co-culturing fermentation, seven different fermentation parameters, including inoculum ratio (v.v^{-1}), overall inoculum size (v.v^{-1}), pH, concentration of the substrate (w.v^{-1}), mixing rate (rpm), temperature, and incubation period were selected after laboratory-scale testing and a literature review (De Vasconcelos et al. 2004; Fadel et al. 2013). The optimization of the selected alcohol fermentation parameters was done using a batch fermentation setup. The optimization process was performed by applying one variable at a time (OVAT) approach in accordance with (De Vasconcelos et al. 2004; Fadel et al. 2013; Hawaz et al. 2023).

Firstly, the effect of the inoculum ratio and overall inoculum size on alcohol production was examined by varying the volume of the inoculum (0/100, 5/95, 20/80, 40/60, 60/40, 80/20, 95/5, and 100/0 v.v^{-1}), and 10–35 (% (v.v^{-1})), respectively (Wu et al. 2023), while keeping all other variables constant. The pH of the fermentation medium was optimized by varying the pH value from 3.5 to 6.5 while the optimum value of the inoculum size and inoculum ratio was maintained. The pH of the fermentation medium was adjusted using 1N NaOH and 99.8% H_2SO_4. The temperature was optimized by adjusting the temperature to 25, 30, 37, 40, and 42 °C, while the pH, inoculum size, and inoculum ratio were kept at their optimum levels. Optimization of the mixing rate was conducted by varying the agitation rate from 50–210 rpm. The molasses concentration was optimized by adjusting the substrate content from 15–40 °brix. Finally, the incubation period was optimized by varying in the range of 24–96 h and keeping all the fermentation parameters at their optimal condition.

Experimental design

The OVAT approach has several limitations (Akalın et al. 2013) and, therefore, response surface methodology (RSM) based on a central composite design (CCD) was used to optimize the bioethanol production during the co-cultivation experiment. Fermentation parameters that significantly affected the bioethanol yield were selected according to the data obtained from the OVAT experiment. In this experiment, experimental runs based on CCD with two levels (-1 and + 1), which were temperature (25 and 35 °C), mixing rate (110 and 150 rpm),

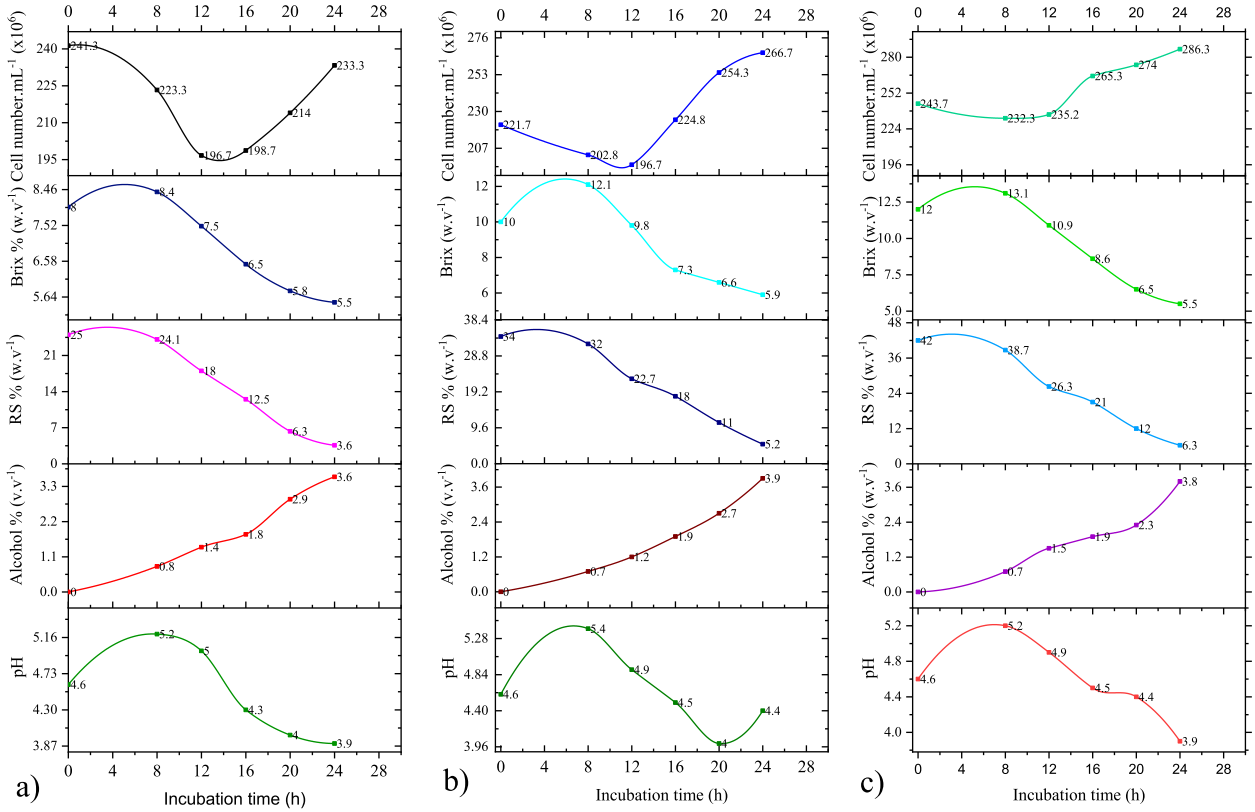

Fig. 2 Propagation dynamics of *S. cerevisiae* isolate TA2 under (**a**) 8 °brix, (**b**) 10 °brix, and (**c**) 12 °brix pH 4.6, temperature 30 °C, DAP concentration 2 g.L^{-1}, and molasses concentration 30 °brix (w.v^{-1}) (conditions taken from the literature). Under the third propagation phase (12 °brix), a viable yeast cell density of 2.86×10^8 cells. mL^{-1} was recorded as an inoculum for fermentation at pH 3.98 at 24 h

Table 1 The level of the experimental design

Parameters	Levels		
	-1	0	+1
Temperature, °C	25	30	35
Molasses concentration, (w.v^{-1})	25	30	35
Mixing rate, rpm	110	130	150
Incubation period, h	48	54	72

molasses concentration (25 and 35 °brix), and incubation time (36 and 72 h) were generated using Design-Expert 12.0 (State-Ease, Inc., Minneapolis, USA) (Table 1) using bioethanol yield (%) as the response variable. In the CCD there were a total of $2^K + 2K + n_0$ experimental combinations, where K denotes the number of independent variables and n_0 is the number of experiential reputations at the center point performed. As a result, 30 experiments were performed (Table 5).

Analysis of Variance (ANOVA) was used to determine the significance of each factor to produce bioethanol in terms of a linear, interactive, and quadratic approach, and is represented using the following second-order quadratic Eq. (1):

$$Y = \beta o + \sum_{i=1}^{3} \beta_i x_i \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j + \sum_{4}^{i=1} \beta_{ii} x_i^2 \quad (1)$$

Where *Y* is the bioethanol yield (%), *βo* the value of the center point. *βi, βij,* and *βii* the linear, interactive, and quadratic coefficients, respectively, and *xi* and *xj* are the independent factors.

Analytical determinations

The concentration of total reducing sugar (TRS%) was determined according to the standard method of Miller (1959) at Wonji Research and Development Center, Ethiopian Sugar Corporation, Wonji, Ethiopia. The ethanol concentration analysis was carried out at the Department of Food Engineering, Addis Ababa Science and Technology University, Ethiopia. The ethanol concentration (g.L^{-1}) was measured by high-performance liquid chromatography (HPLC) (1200 Series Agilent HPLC, Germany) equipped with a UV detector at 280 nm (model Agilent 1260 infinity, Germany) and Spheris orb Amino

(NH_2) Cartridge column (pore size 80A, inner diameter 4.6 mm, length 250 mm, and particle size 5 µm, Waters, Germany). The mobile phase was acetonitrile and water (70:30 ($v.v^{-1}$)), the flow rate was 0.25 mL/min, and the sample injection volume was 10 µl with a column temperature of 25 °C. Estimation of bioethanol yield ($Y_{E/S}$), ethanol productivity (P_V), and fermentation efficiency (FE) was determined using Eqs. 2–4 as described by (Laopaiboon et al. 2009).

$$Y_{E/S\,(\%)} = \frac{bioethanol\ concentration\ (g.L-1)}{total\ sugar\ utilized\ (g.L-1)}\ x\ 100 \quad (2)$$

$$FE\ (\%) = \frac{experimental\ ethanol\ content\ (g.L-1)}{theoretical\ ethano\ lcontent\ (g.L-1)}\ x\ 100 \quad (3)$$

$$PV_{(g/L/h)} = \frac{maximum\ ethanol\ concentration\ (g.L-1)}{fermentation\ time\ (h)} \quad (4)$$

Data analysis

One-way ANOVA (Analysis of variance) was used to analyze the data obtained from the ethanol fermentation trials, and their impact on bioethanol production. Duncan's multiple-range test was applied to separate the means using SPSS version 26. All experiments were done in triplicate.

Results and discussion

Propagation dynamics of the monocultures

The performance of yeast fermentations is determined by its technological properties, including its ability to withstand osmotic stress, oxidative stress, thermic conditions, and/or starvation (Pérez-Torrado et al. 2005). Propagation of the yeast cells was performed with three conditions, each with a different concentration of molasses (i.e., 8, 10, and 12 °brix), which are considered the target ranges of molasses concentrations for an industrial-scale ethanol production process (Hawaz et al. 2022). Figures 1 and 2 demonstrate the dynamics of the propagation for each of the two yeast isolates. The results showed that the pH of the propagation medium increased to alkaline values throughout the propagation phases in the first 8–12 h and 8–16 h for *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively, which is due to the buffering characteristics of sugarcane molasses. Thereafter the pH declined to acidic values at 16 h and 20 h by *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-9, respectively. This suggests that the yeast cells started using the substrate to produce cell biomass by creating favorable conditions inhibiting the growth of competing microbes (Zhang et al. 2019). After 24 h of propagation, *S. cerevisiae* TA2 reduced the pH of the molasses propagation medium (MPM) to 3.9, 4.4, and 3.9 at 8, 10, and 12 °brix, respectively, which is acidic. In contrast, *W. anomalus* HCJ2F-19 reduced the pH with values of pH 4.5, 4.4, and 4.5 at 8, 10, and 12 °brix, respectively, after 24 h. During cell propagation, an acidic pH is created primarily due to the production of organic acids (i.e., acetic acid and lactic acid) and ethanol (the so called the Crabtree effect) by the yeast involved in the propagation process (Dynesen et al. 1998). A reduction of the average pH value from 7.0 to 4.0 was observed during the propagation of *W. anomalus* isolates BT2, BT5, and BT6 in a medium containing sugars, such as sucrose and maltose (Fathiah et al. 2024).

After 24 h of propagation by *S. cerevisiae* TA2, the brix of the MPM declined to 5.5, 5.9, and 5.5 °brix from starting brix values of 8, 10, and 12 °brix, respectively. *W. anomalus* HCJ2F-19 reduced the brix to 4.8, 5.4, and 6.5 °brix from starting values of 8, 10, and 12 °brix, respectively. Like the pH, the brix values increased during the early propagation phase with an average between 8 and 12 h for *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively, and they started to decline after 16 h. Additionally, during the triplicated measurement of the sample, the brix concentration became stable after a 24 h propagation time for all the starting brix (Fig. 1).

During the propagation, the MPM medium was inoculated with a fresh inoculum containing 2.4×10^8 cells. mL^{-1}, and 2.5×10^8 cells. mL^{-1} of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively (Figs. 1 and 2). Both yeast strains showed a reduced cell viability after initial exposure to the propagation medium (Fig. 1). A high concentration of sugars in the molasses, along with the intensive aeration and aerobic metabolism during the initial fed-batch propagation cause osmotic pressure and oxidative stress, which contribute to the decline of the yeast cell population or density (Pérez-Torrado et al. 2005). After 12 h of incubation, the density of the yeast cells began to gradually increase. *W. anomalus* HCJ2F-19 showed a higher viable cell density (i.e., 3.5×10^8 cells. mL^{-1}) compared to *S. cerevisiae* TA2 (i.e., 2.9×10^8 cells. mL^{-1}) at 24 h propagation time. This suggests that the yeast cells reacted to specific stress factors by using their transcriptional factors, which allowed them to quickly adjust to a new environment (Martinez-Pastor et al. 1996). Bellido et al. (2013) reported that *W. anomalus* reached a maximum population of 0.95×10^8 cells. mL^{-1} at day 5. Throughout the entire course of propagation, the density of the yeast cultures increased. *S. cerevisiae* TA2 developed a cell density of 2.3, 2.7, and 2.9×10^8 cells. mL^{-1} at 8, 10, and 12 °brix, respectively, at 24 h. According to Ye et al. (2014), *S. cerevisiae* exhibited a maximum cell population of 3.6×10^8 cells. mL^{-1} in a single species experiment, which is similar to what we observed in the

current study. During the propagation of *S. cerevisiae*, the utilization of non-fermentable carbon sources like ethanol and glycerol enhanced the cell biomass of the yeast (Dobrescu et al. (2021). Moreover, at 8, 10, and 12 °brix, *W. anomalus* HCJ2F-19 achieved a cell count of 1.7, 2.8, and 3.5×10^8 cells. mL^{-1}, respectively at 24 h. Our results showed that *S. cerevisiae* TA2 lowered the concentrations of fermentable sugar to 3.5, 5.2, and 5.5% ($w.v^{-1}$) at 24 h. *W. anomalus* HCJ2F-19 showed a significant decrease of the residual sugar content, with values of 2.2, 3.2, and 3.5%, at 24 h.

Mono-culture fermentations

The fermentation dynamics of the isolates were performed in duplicate under identical growth conditions using MFM as fermentation medium (Figs. 3, and Table 2). Cell density, ethanol yield, ethanol concentration, ethanol productivity, molasses concentration, pH, fermentation efficiency, and residual sugar concentration were the parameters used to evaluate the dynamics of the fermentations. The results showed that the density of the populations declined after an incubation period of 24 h. *S.cerevisiae* TA2 showed a reduction of the yeast numbers from 2.1×10^8 cells. mL^{-1} to 1.8×10^8 cells. mL^{-1} (i.e., 15.1%), 1.4×10^8 cells. mL^{-1} (i.e., 34.5%), and 0.3×10^8 cells. mL^{-1} (i.e., 85.4%) after 24, 48, and 72 h, respectively (Table 2). Previous research has indicated that the concentration of sugar, the availability of nitrogen, and other carbon sources in the fermentation medium affect *S. cerevisiae's* biomass yield (Martínez-Moreno et al. 2012). *W. anomalus* HCJ2F-19 showed a decline in cell numbers from 2.1×10^8 cells. mL^{-1} to 1.8×10^8 cells. mL^{-1} (i.e., 16.0%), 1.5×10^8 cells. mL^{-1} (i.e., 31.1%), and 0.5×10^8 cells. mL^{-1} (i.e., 76.4%) at 24, 48, and 72 h, respectively.

The production of bioethanol increased until the fermentation period reached 48 h and declined thereafter, which might be because the yeast cells enter the stationary phase in which their activity decreased due to factors like depletion of nutrients, accumulation of ethanol, or changes in pH. This in turn can result in a slower rate of sugar consumption and lower ethanol production. On the other hand, after sugars were depleted, ethanol could be reassimilated by the yeasts as a carbon source. Figure 3b demonstrates that at 24 h there was no significant difference ($p > 0.05$) in ethanol production in the single isolate fermentations between *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19. *S. cerevisiae* TA2 produced an

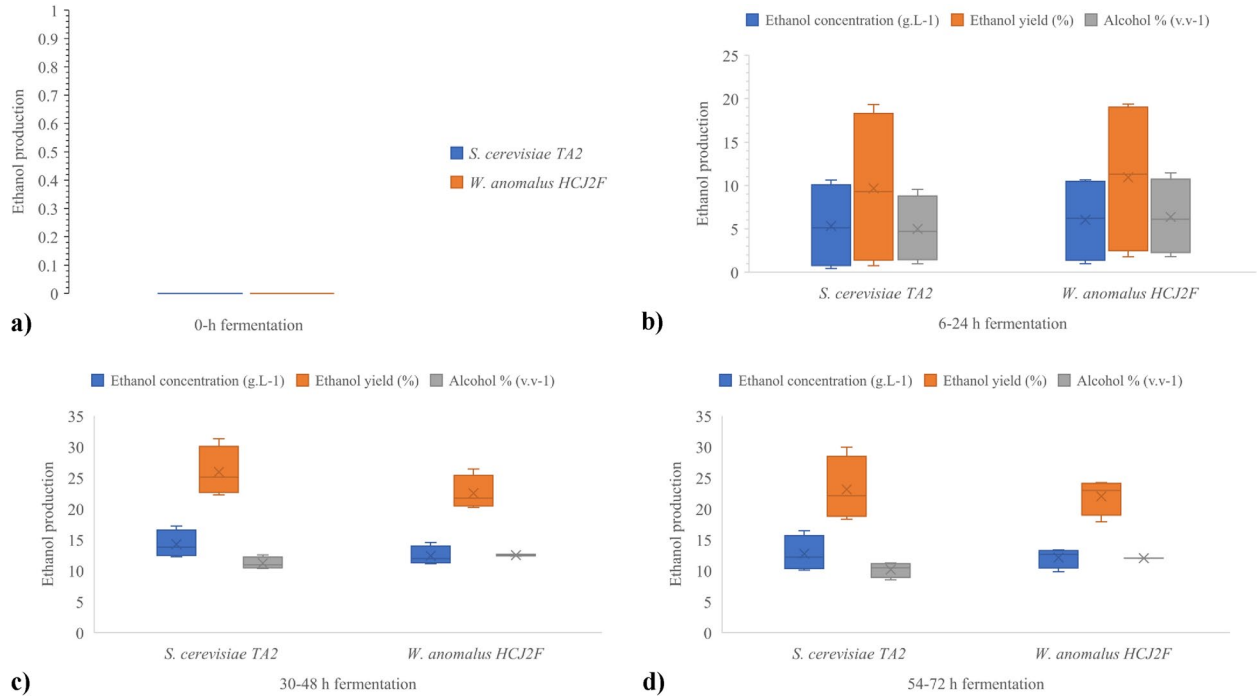

Fig. 3 Box plot analysis of ethanol concentration ($g.L^{-1}$), ethanol yield (%), and alcohol percentage ($v.v^{-1}$) at 72 h fermentation time by *S. cerevisiae* isolates TA2 and *W. anomalus* isolates HCJ2F-19. **a** shows the fermentation dynamics at 0 h after inoculation with essentially minimal data recorded. **b** demonstrate the dynamics of the ethanol production parameters throughout 6 to 24 h. Accordingly, *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 produced ethanol yields of 19.3% and 19.4%, respectively at 24 h. **c** show the fermentation dynamics between 30 and 48 h and the production of a maximum ethanol yield of 31.0% and 26.4% by *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively, at 48 h. **d** illustrates the dynamics of fermentation between 54 and 72 h and shows a decline of all parameters

Table 2 Dynamics of the fermentation of the two yeast monocultures in a laboratory setting using30 °brix, pH 4.6, and 30 °C

Yeast strains	Fermentation time (h)	Ethanol yield ($g.g^{-1}$)	RS ($g.L^{-1}$)	Sugar consumption (%)	Sugar consumption rate ($g.L^{-1}.h^{-1}$)	Fermentation efficiency (%)	Degree brix ($w.v^{-1}$)	pH	Cell viability ($\times 10^6$ cells. mL^{-1})
S. cerevisiae TA2	0	0.00 ± 0.00	53.45 ± 0.00	0.00 ± 0.03	0.00 ± 0.00	0.00 ± 0.00	30 ± 0.00	4.60 ± 0.00	206.00 ± 0.00
	6	0.01 ± 0.00	49.85 ± 0.00	6.74 ± 0.06	0.60 ± 0.00	1.62 ± 0.06	29.59 ± 0.04	4.85 ± 0.00	196.00 ± 0.03
	12	0.04 ± 0.01	38.54 ± 0.01	27.90 ± 0.02	1.24 ± 0.02	7.00 ± 0.03	27.44 ± 0.01	4.74 ± 0.00	188.67 ± 0.03
	18	0.16 ± 0.00	8.03 ± 0.01	84.98 ± 0.01	2.52 ± 0.03	32.32 ± 0.09	16.65 ± 0.00	4.65 ± 0.00	181.33 ± 0.06
	24	0.20 ± 0.00	7.66 ± 0.00	85.67 ± 0.01	1.91 ± 0.00	40.86 ± 0.00	14.23 ± 0.00	3.92 ± 0.00	175.00 ± 0.02
	30	0.24 ± 0.00	7.04 ± 0.00	86.83 ± 0.03	1.55 ± 0.01	47.00 ± 0.00	14.05 ± 0.02	3.94 ± 0.00	158.00 ± 0.02
	36	0.25 ± 0.00	6.98 ± 0.00	86.94 ± 0.00	1.29 ± 0.00	50.46 ± 0.15	14.01 ± 0.00	3.94 ± 0.00	153.67 ± 0.00
	42	0.28 ± 0.00	5.49 ± 0.05	89.73 ± 0.04	1.14 ± 0.06	55.64 ± 0.03	13.44 ± 0.00	3.94 ± 0.00	148.00 ± 0.03
	48	0.33 ± 0.00	2.56 ± 0.00	95.21 ± 0.00	1.06 ± 0.05	66.22 ± 0.19	12.33 ± 0.01	3.95 ± 0.00	135.00 ± 0.01
	54	0.32 ± 0.00	2.56 ± 0.00	95.21 ± 0.00	0.94 ± 0.02	63.31 ± 0.00	11.97 ± 0.00	3.95 ± 0.00	112.00 ± 0.02
	60	0.25 ± 0.00	2.56 ± 0.00	95.21 ± 0.00	0.85 ± 0.01	50.83 ± 0.00	12.02 ± 0.02	3.95 ± 0.00	101.00 ± 0.00
	66	0.21 ± 0.00	2.56 ± 0.00	95.21 ± 0.03	0.77 ± 0.02	42.73 ± 0.02	12.02 ± 0.00	3.95 ± 0.00	53.33 ± 0.05
	72	0.19 ± 0.00	2.56 ± 0.00	95.21 ± 0.00	0.71 ± 0.02	38.71 ± 0.02	12.02 ± 0.00	3.95 ± 0.00	0.30 ± 0.01
W. anomalus HCJ2F-19	0	0.00 ± 0.00	53.45 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	30.00 ± 0.00	4.60 ± 0.00	212.00 ± 0.02
	6	0.02 ± 0.01	39.55 ± 0.00	26.01 ± 0.02	2.32 ± 0.01	3.77 ± 0.03	28.00 ± 0.01	4.75 ± 0.00	202.00 ± 0.03
	12	0.05 ± 0.00	22.00 ± 0.00	58.84 ± 0.03	2.62 ± 0.02	9.81 ± 0.00	23.22 ± 0.05	4.65 ± 0.00	198.00 ± 0.00
	18	0.19 ± 0.00	5.55 ± 0.00	89.62 ± 0.03	2.66 ± 0.00	38.00 ± 0.05	15.33 ± 0.03	4.45 ± 0.00	186.00 ± 0.02
	24	0.20 ± 0.00	4.32 ± 0.00	91.92 ± 0.02	2.05 ± 0.00	40.99 ± 0.00	14.25 ± 0.00	4.12 ± 0.00	178.67 ± 0.01
	30	0.21 ± 0.00	3.22 ± 0.00	93.98 ± 0.00	1.67 ± 0.00	42.73 ± 0.00	13.11 ± 0.01	3.94 ± 0.00	171.00 ± 0.03
	36	0.22 ± 0.00	2.88 ± 0.01	94.61 ± 0.00	1.40 ± 0.03	44.88 ± 0.00	13.05 ± 0.02	3.85 ± 0.00	164.67 ± 0.02
	42	0.24 ± 0.00	2.44 ± 0.00	95.43 ± 0.01	1.21 ± 0.02	47.00 ± 0.00	13.00 ± 0.03	3.75 ± 0.00	155.00 ± 0.03
	48	0.28 ± 0.00	1.56 ± 0.00	97.08 ± 0.00	1.08 ± 0.03	55.86 ± 0.02	12.04 ± 0.00	3.65 ± 0.01	146.00 ± 0.02
	54	0.26 ± 0.00	1.56 ± 0.01	97.08 ± 0.04	0.96 ± 0.03	51.27 ± 0.00	10.33 ± 0.00	3.65 ± 0.00	132.00 ± 0.01
	60	0.25 ± 0.01	0.22 ± 0.00	99.59 ± 0.03	0.89 ± 0.01	50.49 ± 0.00	8.34 ± 0.02	3.65 ± 0.00	108.33 ± 0.04
	66	0.20 ± 0.02	0.19 ± 0.00	99.64 ± 0.02	0.81 ± 0.01	47.00 ± 0.05	8.26 ± 0.01	3.65 ± 0.00	81.00 ± 0.00
	72	0.18 ± 0.02	0.14 ± 0.00	99.74 ± 0.04	0.74 ± 0.01	37.81 ± 0.05	8.33 ± 0.03	3.65 ± 0.00	0.50 ± 0.00

ethanol concentration of 10.6 $g.L^{-1}$, an ethanol yield of 0.20 $g.g^{-1}$, and 19.3%, and an alcohol percentage ($v.v^{-1}$) of 9.5. In agreement with our findings, an ethanol concentration of 7.5 $g.L^{-1}$, a yield of 0.3 $g.g^{-1}$, and a productivity of 0.20 $g{\cdot}L^{-1}.h^{-1}$ was produced by *S. cerevisiae* isolate OVB11 after 36 h of fermentation from a biomass containing glucose (Yadav et al. 2011). *S. cerevisiae* strain H28 produced ethanol 7.6 $g.L^{-1}$ at 48 h incubation (Naito et al. 2019). *W. anomalus* HCJ2F-19 formed at 24 h 10.7 $g.L^{-1}$ ethanol, an ethanol yield of 0.20 $g.g^{-1}$ and 19.8%, and an alcohol percentage of 11.5%., At 48 h of fermentation, the *S. cerevisiae* TA2 produced a similar amount of bioethanol (viz., 17.0 $g.L^{-1}$) compared to *W. anomalus* HCJ2F-19 that produced 15.0 $g.L^{-1}$ (Fig. 3c). Similar to our finding, Naito et al. (2019) reported that *W. anomalus* strain AN2-56 produced 13.9 $g.L^{-1}$ of ethanol using a glucose containing substrate after 48 h of fermentation. Another study reported that *W. anomalus* isolate M15 produced 5.8 $g.L^{-1}$ ethanol using glucose as a substrate, which is a lower yield than our currently observed value (Turner et al. 2022). According to Henderson et al. (2013), an average ethanol concentration ranging from 8.0 to 10.7 $g.L^{-1}$ was obtained by different *Saccharomyces* isolates using grape biomass as a substrate. These lower final ethanol concentrations may have resulted from a lower tolerance of the isolates used to various stress conditions, such as acidic pH, ethanol content, and other fermentation-related factors (Henderson et al. 2013).

Saccharomyces cerevisiae TA2 produced an ethanol yield of 0.33 $g.g^{-1}$ and 31.3%, 17.2 $g.L^{-1}$ ethanol, 66.2% of fermentation efficiency, and 0.36 $g.L^{-1}$ h^{-1} of productivity at a yeast viability of 1.4×10^6 cells.mL^{-1} at 48 h. *W. anomalus* HCJ2F-19 yielded an ethanol content of 0.28 $g.g^{-1}$ and 26.4%, 14.5 $g.L^{-1}$ ethanol, an fermentation efficiency of 55.9%, and productivity of 0.30 $g.L^{-1}.h^{-1}$ at a cell density of 1.5×10^6 cells.mL^{-1} at 48 h. Compared to the currently observed values, *W. anomalus* isolate BT6 showed an ethanol production of 5.0 $g.L^{-1}$, an ethanol productivity of 0.10 $g.L^{-1}.h^{-1}$, and a fermentation efficiency of 80.6%, respectively after 48 h of incubation

at 30 °C (Fathiah et al. 2024). The bioethanol production and fermentation efficiency of both yeast isolates dropped when the fermentation duration was extended to 72 h (Fig. 3d). This could be due to the depletion of fermentable sugars present in the substrate as well as the presence of stressful conditions, including an acidic pH and high ethanol concentrations (Fathiah et al. 2024; Yadav et al. 2011) (Table 2).

Both yeast isolates were reported to be acid-tolerant (Hawaz et al. 2022). Regarding the impact of the pH during the fermentations, an ethanol yield of 0.33 $g.g^{-1}$ at a pH of 3.9, and 0.26 $g.g^{-1}$ at a more acidic pH of 3.7 was produced by *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively (Table 2). Furthermore, a strong positive correlation was observed between the reduction in molasses concentration and the amount of reduced sugar. *S. cerevisiae* TA2 produced a maximum ethanol concentration of 17.2 $g.L^{-1}$ with a yield of 31.3% at 48 h after consuming 95% of the substrate. On the other hand, *W. anomalus* HCJ2F-19 consumed 97% of the reducing sugar to produce 14.5 $g.L^{-1}$ ethanol and 26.4% ethanol yield, respectively. Fathiah et al. (2024) revealed that *W. anomalus* isolates BT2 and BT6 showed a substrate consumption rate of 61% and 60%, respectively, from glucose and maltose containing biomass at 48 h of fermentation.

Co-fermentation of S. cerevisiae TA2 and W. anomalus HCJ2F-19

The dynamics of the co-fermentation of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 are presented in Fig. 4 and Table 3. The synergistic effect of the co-culturing was investigated by comparing it with the fermentation dynamics of the mono-culture fermentations of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19. In the current investigation, co-culturing of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 produced a higher bioethanol yield than the monocultures of both strains, namely 22.2 g. L^{-1} for the co-culturing versus 17.2 $g.L^{-1}$ and 14.5 g. L^{-1}, for monocultures (*S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively) (Table 3). The results also revealed that the overall fermentation time required for co-culturing was 36 h which is significantly shorter than it was for mono-culturing that used 48 h. This indicates that the mixed cultures effectively fermented sugars resulting from possible synergistic effect and complementary traits of the two yeast strains. In addition, the co-culture fermentation required 36 h to produce a bioethanol concentration of 22.2 $g.L^{-1}$ with a yield of 40.4% and an amount of 0.43 $g.g^{-1}$ (Figs. 4a and b), whereas the monoculture of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 required 48 h to produce a bioethanol concentration of

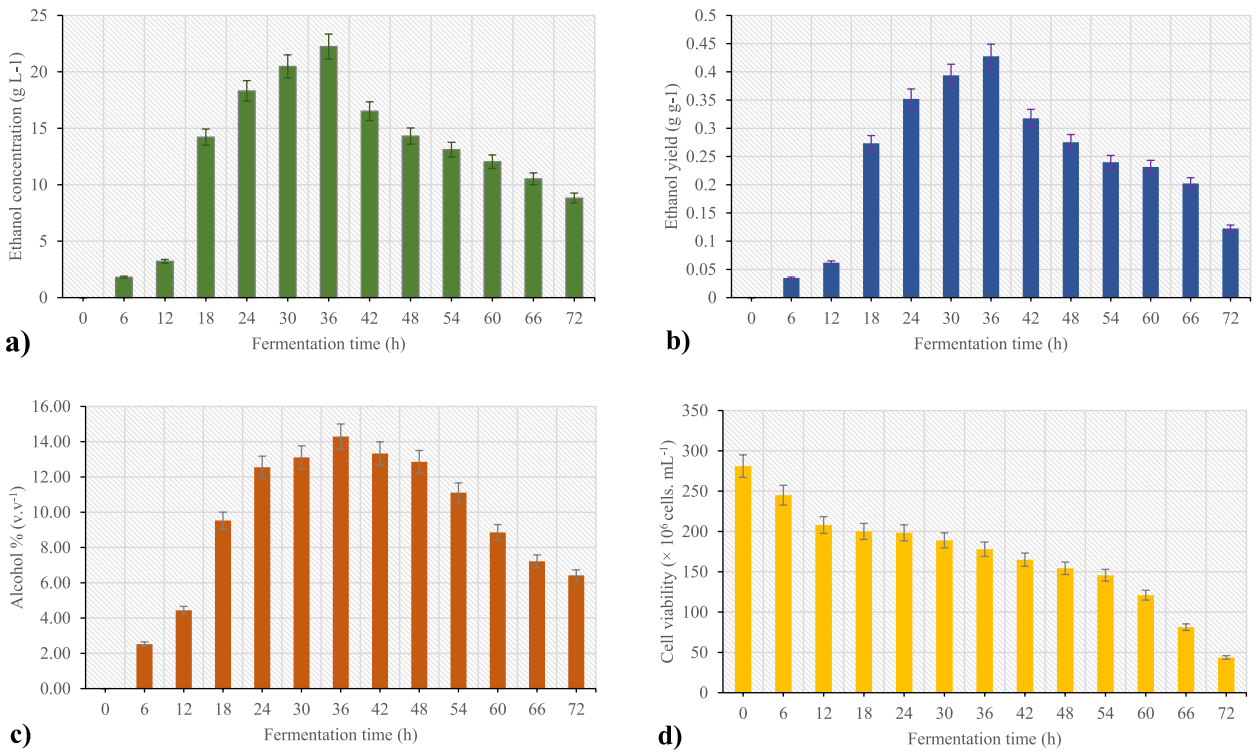

Fig. 4 The dynamics of co-culture fermentation of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 up to 72 h. In all parameters, the maximum ethanol production was recorded after 36 h. **a** bioethanol concentration ($g.L^{-1}$), (**b**) bioethanol yield ($g.g^{-1}$), (**c**) alcohol percent ($v.v^{-1}$), and (**d**) cell viability ($\times 10^6$ cells. mL^{-1})

Table 3 Dynamics of the co-cultures fermentation in a laboratory setting at 30 °brix, pH 4.6, and 30 °C

Yeast strains	Fermentation time (h)	Ethanol yield ($g.g^{-1}$)	RS ($g.L^{-1}$)	Sugar consumption (%)	Sugar consumption rate ($g.L^{-1}.h^{-1}$)	Fermentation efficiency (%)	Degree brix ($w.v^{-1}$)	pH	Cell viability ($\times 10^6$ cells. mL^{-1})
S. cerevisiae TA2/*W. anomalus* HCJ2F-19	0	0.00 ± 0.00	53.45 ± 0.01	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.00	30.00 ± 0.00	4.60 ± 0.01	281.00 ± 0.00
	6	0.03 ± 0.03	42.08 ± 0.01	21.27 ± 0.01	1.90 ± 0.01	6.81 ± 0.03	20.22 ± 0.01	4.85 ± 0.01	245.00 ± 0.01
	12	0.06 ± 0.02	32.05 ± 0.01	40.04 ± 0.04	1.78 ± 0.01	12.11 ± 0.03	19.21 ± 0.01	4.65 ± 0.00	208.00 ± 0.01
	18	0.27 ± 0.03	4.44 ± 0.01	91.69 ± 0.01	2.72 ± 0.00	53.46 ± 0.00	15.32 ± 0.01	4.55 ± 0.00	200.00 ± 0.01
	24	0.35 ± 0.00	2.54 ± 0.00	95.25 ± 0.00	2.12 ± 0.02	68.84 ± 0.00	13.33 ± 0.01	4.22 ± 0.01	198.33 ± 0.02
	30	0.39 ± 0.00	1.86 ± 0.00	96.52 ± 0.01	1.72 ± 0.05	77.00 ± 0.02	12.12 ± 0.01	4.06 ± 0.01	189.00 ± 0.03
	36	0.43 ± 0.00	1.52 ± 0.03	97.16 ± 0.01	1.44 ± 0.01	83.60 ± 0.02	12.01 ± 0.00	3.95 ± 0.01	178.00 ± 0.01
	42	0.32 ± 0.00	1.84 ± 0.02	96.56 ± 0.00	1.23 ± 0.04	62.11 ± 0.01	11.22 ± 0.00	3.75 ± 0.01	165.00 ± 0.01
	48	0.28 ± 0.03	2.85 ± 0.02	94.67 ± 0.00	1.05 ± 0.01	53.81 ± 0.03	10.32 ± 0.01	3.65 ± 0.01	156.33 ± 0.00
	54	0.25 ± 0.05	2.85 ± 0.033	94.67 ± 0.00	0.94 ± 0.01	49.29 ± 0.04	8.33 ± 0.01	3.65 ± 0.01	145.67 ± 0.00
	60	0.23 ± 0.02	2.85 ± 0.00	94.67 ± 0.01	0.84 ± 0.01	45.27 ± 0.00	8.33 ± 0.01	3.60 ± 0.00	121.00 ± 0.00
	66	0.20 ± 0.01	2.85 ± 0.00	94.67 ± 0.01	0.77 ± 0.00	39.55 ± 0.00	8.33 ± 0.00	3.65 ± 0.00	81.33 ± 0.03
	72	0.17 ± 0.03	2.85 ± 0.01	94.67 ± 0.01	0.70 ± 0.02	33.15 ± 0.02	8.33 ± 0.00	3.65 ± 0.00	0.60 ± 0.01

17.2 $g.L^{-1}$ and 14.5 $g.L^{-1}$, and ethanol yield 0.33 $g.g^{-1}$ and 0.28 $g.g^{-1}$, respectively. Yadav et al. (2011) investigated ethanol fermentation using a co-culture of *S. cerevisiae* isolate OVB11 and *P. stipitis* isolate NCIM3498. The results showed an ethanol concentration of 12 g. L^{-1} with an efficiency of 95%, a volumetric productivity of 0.33 $g.L^{-1}.h^{-1}$, and a yield of 0.4 $g.g^{-1}$ after 36 h. In agreement with the current finding, co-culturing of *S. cerevisiae* ITV-01 and *P. stipitis* NRRL Y-7124 showed a fivefold improvement of ethanol productivity compared to the monoculture fermentations (Gutiérrez-Rivera et al. 2012). Similar to this, coculturing of *S. cerevisiae* MTCC 174 and *S. stipitis* NCIM 3497 (formerly *P. stipitis*) resulted in a higher maximum ethanol concentration of 20.8 $g.L^{-1}$ when compared to *S. cerevisiae* MTCC 174 (14.0 $g.L^{-1}$) and *S. stipitis* NCIM No. 3497 (12.2 $g.L^{-1}$) alone (Singh et al. 2014). Likewise, more ethanol was produced when coculturing *S. cerevisiae* ATCC 26602 and *S. stipitis* DSM 3651 that yielded 7.4 $g.L^{-1}$ ethanol, when compared to the monoculture of *S. cerevisiae* that gave 6.7 $g.L^{-1}$ ethanol (Karagöz & Özkan 2014). According to Wu et al. (2023) an ethanol concentration of 15.4 g. L^{-1} was obtained at the end of a co-culturing fermentation with *S. stipites* and *S. cerevisiae,* a value that is lower than observed in the current study. In the present study, an increase of 29% and 53% of the bioethanol yield was obtained during co-fermentation in about 12 h when compared to the monocultures of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively. Overall, the results demonstrated a superior bioethanol fermentation performance of the co-culturing over the mono-culturing of the two yeast species isolates. This might be due because of nutrient utilization during the co-fermentation ensuring a healthy yeast cell population throughout the fermentation process leading to a higher ethanol production as was also suggested by Zohre and Erten (2002).

However, it is needed to further define the growth state of each isolate to determine whether competition or synergy occurred between *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 throughout the co-culturing process. During the first co-fermentation phase, the cell density and cell abundance of both yeast isolates dropped to 2.0×10^8 cells. mL^{-1} at 24 h, respectively, which can be attributed to the high concentration of sugar, the presence of organic molecules that act as inhibitors, and other growth-inhibiting compounds present in sugarcane molasses such as the metal ions (Phisalaphong et al. 2006). After sequential incubation, the cell density of the coculture significantly declined to 1.6×10^8 cells. mL^{-1}, and 0.6×10^6 cells. mL^{-1} at 48 h and 72 h, respectively, which can be explained by inhibition because of the accumulation of ethanol and the acidic pH of the fermentation medium (Yadav et al. 2011). Other factors that could contribute to the decline in viability may include cell–cell interactions, nutritional depletion, limited oxygen availability, and quorum sensing (Nissen & Arneborg 2003). The relatively good cell viability during the mono and co-fermentations suggests good stress-tolerant properties of the yeast strains. The growth nor its existence of both strains was impacted by the other. Our result is partially in agreement with the findings of Zohre and Erten (2002) who observed that the growth of *S. cerevisiae* was not impacted by the presence of non-*Saccharomyces* yeasts during the fermentation of grape juice. Other research

findings demonstrated that the population of *W. anomalus* dropped rapidly after the inoculation of *S. cerevisiae* (Lee et al. 2010; Mendoza et al. 2007). In our work, *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 reached a maximum value of 1.4×10^8 cells. mL^{-1}, and 1.5×10^8 cells. mL^{-1} at 48 h, respectively, while, in the coculture they had higher robustness and attained 1.6×10^8 cells. mL^{-1}. In the co-culture fermentation a bioethanol productivity of 1.44 $g.L^{-1}.h^{-1}$ and a corresponding ethanol concentration 22.2 $g.L^{-1}$ was achieved at a cell density of 1.8×10^8 cells. mL^{-1} at 36 h (Fig. 4c and d). Moreover, co-culturing of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 produced a higher fermentation efficiency of 83.6% compared to mono-culturing with values of 66.2% and 55.9% for *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19, respectively. Similarly, Aswathy et al. (2010) reported that a co-culture fermentation of *S. cerevisiae* and *C. tropicalis* produced the highest amount of ethanol when compared to the monocultures of *S. cerevisiae* and *C. tropicalis*. The effect of complete conversion of sugars into ethanol resulted in an increased ethanol production and fermentation efficiency (Yadav et al. 2011).

In the present study, *S. cerevisiae* TA2 showed a comparative high sugar consumption rate during the first co-fermentation phase with rates of 2.72 $g.L^{-1}.h^{-1}$, and 2.12 $g.L^{-1}.h^{-1}$ at 18 at 24 h, respectively, while in the first phase of the co-fermentation, the sugar consumption rate was slow (Table 3). On the hand, the simultaneous inoculation did not affect the fermentation efficiency of the mixed culture since all the fermentations resulted in low amounts of residual sugar, i.e., 1.5 $g.L^{-1}$. A comparison of the fermentation performances of mono-culturing and co-culturing of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 is presented in Table 4. During the co-fermentation an ethanol concentration of 22.24 $g.L^{-1}$ was achieved at 36 h of fermentation.

Optimization of fermentation conditions

Firstly, the effect of pH on the alcohol production was assessed by altering the pH of the medium in accordance with the methodology, while other parameters were maintained constant (De Vasconcelos et al. 2004; Fadel et al. 2013) (Fig. 5f). As a result, a pH value of 5.0 yielded a 14.3% alcohol content. It has been reported that the best pH for yeast fermentations is 4.0–5.0 (Lin et al. 2012). In addition, pH values below 4.0 and above 5.0 enhance the formation of acetic acid, and butyric acid during ethanol fermentation, respectively (Lin et al. 2012). Hu et al. (2012) reported a wide range of optimum pH values (viz., 4.0–8.0) during fermentation of Jerusalem artichoke tuber by *S. cerevisiae* isolate JZ1C. After optimization of the pH, the molasses concentration ($w.v^{-1}$) was optimized using the optimal pH 5.0, and as a result 14.4% ethanol was produced using 25 °brix of molasses (Fig. 5e), showing that the twin-consortium was able to resist high sugar concentrations, which would boost the production of bioethanol in the end and result in greater energy savings during downstream processes (Mukasekuru et al. 2020). In addition, Hawaz et al. (2022) reported that *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 showed high sugar (40% $w.v^{-1}$) and ethanol tolerances (i.e., 18% $v.v^{-1}$), and the high concentrations of sugar and ethanol in the medium may have a positive effect on the alcohol production throughout the co-culturing process (Du Preez et al. 1987; Liang et al. 2013).

The influence of the overall inoculation size and inoculum ratio on the co-culture fermentation was further optimized by varying the inoculum size and inoculum ratio throughout the experiment (Figs. 5c and g). Our results revealed that a maximum alcohol percentage of 14.1% was realized at conditions of 10% of overall inoculum size and a 60:40 inoculum ratio of *S. cerevisiae* TA2/*W. anomalus* HCJ2F-19. In agreement with our findings, Wu et al. (2023) found the highest substrate conversion rate to ethanol with an inoculum size of 10% and inoculum ratio of 60:40 of *S. cerevisiae/S. stipitis*, respectively.

The effect of temperature on the synergistic impact of the co-fermenting consortium on the ethanol fermentation was evaluated using pH 5.5, 25 °brix, 10% inoculum

Table 4 Comparison of fermentation performances of mono-culturing and co-culturing of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19

Yeast strains	Fermentation time (h)	Ethanol concentration ($g.L^{-1}$)	Ethanol yield ($g.g^{-1}$)	Sugar consumption (%)	Sugar consumption rate ($g.L^{-1}.h^{-1}$)	Fermentation efficiency (%)	Ethanol Productivity ($g.L^{-1}.h^{-1}$)
S. cerevisiae TA2	48	17.22 ± 0.02^b	0.33 ± 0.00^b	95.22 ± 0.00^b	1.06 ± 0.02^b	66.22 ± 0.02^b	0.36 ± 0.03^b
W. anomalus HCJ2F-19	48	14.52 ± 0.01^c	0.28 ± 0.02^c	97.08 ± 0.02^a	1.08 ± 0.00^b	55.86 ± 0.03^c	0.30 ± 0.02^c
S. cerevisiae TA2/*W. anomalus* HCJ2F-19	36	22.24 ± 0.03^a	0.43 ± 0.01^a	97.20 ± 0.01^a	1.44 ± 0.01^a	83.60 ± 0.02^a	0.62 ± 0.05^a

NB Different letters in superscripts along columns indicate a significant difference ($p<0.05$), but the same letters indicate no significance

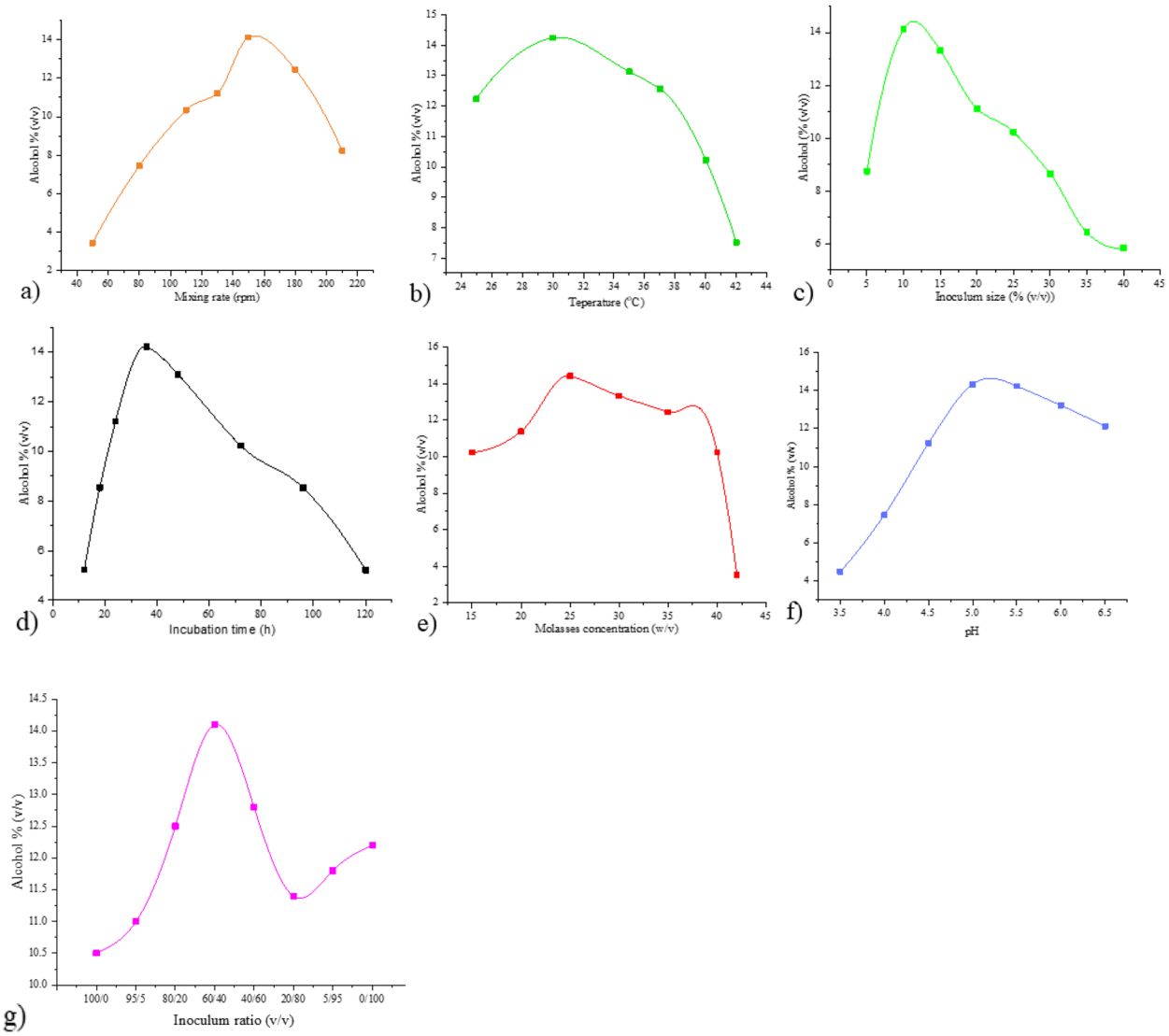

Fig. 5 Optimum alcohol production conditions of co-culturing *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 using batch fermentation system denoted as a function of (**a**) mixing rate, (**b**) temperature, (**c**) inoculum size, (**d**) incubation period, (**e**) molasses concentration, and (**f**) pH

size, and a 60:40 inoculum ratio (Fig. 5b). As shown in Fig. 3b, an alcohol percentage of 14.2% was obtained at 30 °C, but at higher temperatures a fast decline of alcohol content was observed. The optimal temperature for yeast fermentation is between 30 and 35 °C (Hu et al. 2012). Temperature has a considerable impact on a yeast cell's enzymatic activity and membrane turgidity, thus yeasts that are both active and heat-tolerant are appropriate for use in commercial bioethanol production. In addition, it was observed that higher temperatures significantly decrease the exponential phase of the yeast cell population which directly reduced ethanol production (Lin et al. 2012) that could be attributed to the accumulation of toxins, including ethanol, in the yeast cell (Lin et al. 2012).

Using the aforementioned optimal ethanol fermentation conditions, optimization of the mixing rate (rpm) was performed using an orbital shaker controlled at various speeds (Fig. 5a). The alcohol percentage increased as the mixing rate increased from 100 to 150 rpm; however, after 150 rpm, it began to fall. The highest alcohol percentage obtained was 14.1% at 150 rpm, indicating that this condition was optimal. Finally, the incubation period (h) was optimized using all the optimized parameters, i.e., pH 5.5, molasses concentration 25 °brix, temperature 30 °C, mixing rate 150 rpm, and overall inoculum size 10%, and inoculum ratio 60:40 (Fig. 5c). The co-fermenting *S. cerevisiae* TA2/*W. anomalus* HCJ2F-19 produced 14.2% ($v.v^{-1}$) alcohol at 36 h of fermentation time.

Controlled batch fermentations in a benchtop bioreactor with a working volume of 5 L were carried out to experimentally validate all the above-mentioned optimal fermentation conditions, i.e., pH 5.0, molasses concentration 25 ºbrix, temperature 30 °C, mixing rate 150 rpm, overall inoculum size 10%, inoculum ratio 60:40, and an incubation period of 36 h. In this experiment, co-fermenting *S. cerevisiae* TA2/*W. anomalus* HCJ2F-19 produced an ethanol concentration of 26.5 $g.L^{-1}$, ethanol yields of 53% and 0.46 $g.g^{-1}$, a productivity of 0.55 $g.L^{-1}.h^{-1}$, and a fermentation efficiency of 99.5%. Compared to the single culture ethanol fermentations, the ethanol yield of the dual fermentation increased by around 31%. Furthermore, an increase of 69.2% and 100.5% in ethanol yield were observed compared to fermentations using single cultures of *S. cerevisiae* TA2, and *W. anomalus* HCJ2F-19, respectively.

Experimental design

The following four parameters were optimized to maximize bioethanol production using CCD design: molasses concentration, temperature, incubation time, and mixing rate. The CCD generated a matrix of 30 experimental runs, each having a combination of the four selected parameters (mixing rate-A, temperature-B, molasses concentration-C, and incubation time-D) to optimize the bioethanol yield (Table 5). The entire design matrix, along with the predicted and experimental responses, is presented in Table 5. The ethanol yields were significantly and positively influenced by the linear model

Table 5 Complete design matrix in terms of predicted and actual responses using Response surface-central composite design (CCD)

Run	Mixing rate A	Temperature B	Molasses concentration C	Incubation time D	Bioethanol concentration ($g.L^{-1}$)		Bioethanol yield (%)	
					Actual	Predicted	Actual	Predicted
1	105	35	35	36	17.58	16.17	33.81	31.09
2	105	25	35	72	16.92	15.64	32.53	30.08
3	155	25	25	36	17.00	17.56	32.69	33.77
4	130	30	30	54	29.38	28.95	56.49	55.67
5	80	30	30	54	13.00	12.91	25.00	24.82
6	130	30	20	54	22.00	21.11	42.31	40.61
7	130	30	30	54	28.77	28.95	55.32	55.67
8	130	20	30	54	21.00	19.89	40.39	38.26
9	105	35	25	72	17.00	17.78	32.69	34.19
10	155	25	35	72	19.00	20.51	36.54	39.44
11	180	30	30	54	22.75	22.11	43.75	42.53
12	155	35	25	72	21.00	20.23	40.39	38.91
13	130	30	30	90	12.00	11.06	23.08	21.27
14	105	35	25	36	17.00	16.70	32.69	32.11
15	155	25	25	72	19.00	19.93	36.54	38.34
16	155	35	35	72	18.00	17.91	34.62	34.44
17	105	25	25	72	15.00	15.88	28.85	30.55
18	130	30	30	54	28.00	28.95	53.85	55.67
19	105	25	25	36	12.00	11.61	23.08	22.34
20	105	25	35	36	12.00	13.98	23.08	26.89
21	155	25	35	36	22.00	20.74	42.31	39.89
22	130	30	30	54	28.66	28.95	55.11	55.67
23	130	30	40	54	21.00	21.16	40.39	40.69
24	130	40	30	54	22.00	22.38	42.31	43.04
25	130	30	30	54	28.89	28.95	55.56	55.67
26	155	35	35	36	21.00	21.33	40.39	41.02
27	130	30	30	54	30.00	28.95	57.69	55.67
28	130	30	30	18	10.00	10.21	19.23	19.64
29	155	35	25	36	20.25	21.05	38.94	40.47
30	105	35	35	72	14.00	14.65	26.92	28.16

(mixing rate, followed by temperature, incubation time, and molasses concentration), the quadratic model (mixing rate, molasses concentration, temperature), and interaction (molasses concentration, and incubation period) (Eq. 5). The linear mixing rate contributed a highly positive impact on the response, i.e., the bioethanol yield.

$$\begin{aligned}\text{Bioethanolyield (\%)} = {} & +55.67 + 4.43A + 1.19B \\ & + 0.023C + 0.4080D - 0.7692AB \\ & + 0.3920AC - 0.9108AD - 1.39BC \\ & - 1.53BD - 1.25CD - 5.50A^2 \\ & - 3.76B^2 - 3.76C^2 - 8.80D^2 \end{aligned} \quad (5)$$

The ethanol yield obtained from these experimental runs ranged approximately between 19.6 and 55.7%. The coefficient of determination analysis was used to determine the accuracy of the regression polynomial equation (R^2). R^2 values between 0.70 and 1 represent a good model. The fitness of the quadratic models was assessed by comparing the predicted R^2 value with the adjusted R^2 value. As a result, the predicted R^2 value of 0.8792 is in reasonable agreement with the adjusted R^2 value of 0.9772, i.e., the difference is less than 0.2 demonstrating rational agreement between both regression coefficients. The lack-of-fit ($P=0.0628$) of the model is not significant relative to the pure error indicates the non-significance of error in the model. The significance of R^2 was further confirmed using the F value. The model F value of 53.54 indicates that the model used was significant. El-Mekkawi et al. (2019) showed that the statistical analysis of the response variables revealed that R^2, the predicted R^2, and adjusted R^2 were 0.998, 0.992, and 0.997, respectively. The probability of the F function for each model term is less than 0.05 which indicates the significance of all the model terms used, while the probability of the F function for the lack of fit is greater than 0.05 which indicates the non-significance of error. Linear (A and B), interaction (AB, BC, and CD), and quadratic (A^2, B^2, C^2, and D^2) were found significant model terms ($P<0.05$) (Table 6). The model has an adequate precision ratio of 23.3097 which indicates an adequate signal, and this model can thus be used to navigate the design space.

The effects of independent factors on ethanol yield (%) are illustrated by 3D response surface plots obtained after 30 experimental trials (Fig. 6). In general, the 3D surface plot demonstrates that a maximum average ethanol yield of 45.9% was attained. This analysis revealed a positive interactive effect such that the change of the mixing rate from 105.3 to 150.3 rpm, and the temperature from 25.1 to 32.3 improved the ethanol yield from 40.2 to 55.5% (Fig. 6a), indicating a 38% estimated increase of the ethanol yield. Beyond 33 °C and 151 rpm, the ethanol yield

Table 6 Analysis of variance (ANOVA) for bioethanol production of co-fermenting *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19 using CCD experiments

Source	SS	Df	MS	F-value	*p*-value	
Model	3455.78	14	246.84	45.94	< 0.0001	Significant
A-Mixing rate	470.58	1	470.58	87.59	< 0.0001	
B-Temperature	34.24	1	34.24	6.37	0.0233	
C-Molasses	0.0099	1	0.0099	0.0018	0.9663	
D-Incubation time	4.00	1	4.00	0.7437	0.4021	
AB	9.47	1	9.47	1.76	0.2042	
AC	2.46	1	2.46	0.4577	0.5090	
AD	13.27	1	13.27	2.47	0.1369	
BC	31.00	1	31.00	5.77	0.0297	
BD	37.54	1	37.54	6.99	0.0184	
CD	25.12	1	25.12	4.68	0.0472	
A^2	829.50	1	829.50	154.39	< 0.0001	
B^2	386.86	1	386.86	72.00	< 0.0001	
C^2	386.86	1	386.86	72.00	< 0.0001	
D^2	2126.14	1	2126.14	395.73	< 0.0001	
Residual	80.59	15	5.37			
Lack of Fit	72.08	10	7.21	4.23	0.0624	Not significant
Pure Error	8.51	5	1.70			
Total	3536.38	29				

$R^2 = 0.9772$; adjusted $R^2 = 0.9559$; predicted $R^2 = 0.8792$; Adeq. Precision = 38.42

SS Sum of squares, *df* degree of freedom, *MS* Mean square

started to decrease. However, the ethanol yield was not significantly impacted by the interaction effect of temperature and mixing rate ($P=0.2033$) (Table 6). This study also shows that the ethanol yield increased and reached its maximum yield of 56.6% when the initial molasses concentration and mixing rate increased to 31.3 °brix, and 139.9 rpm, respectively (Fig. 6b).

The elliptical shape of Fig. 6c shows that the ethanol yield increased with the increment in the positive interaction between incubation time and mixing rate and recorded its maximum value of 56.4% ethanol within 56.1 h of incubation period and using 143.3 rpm. The ethanol yield decreased with a longer incubation period and a higher mixing rate. Moreover, the bioethanol production was not significantly affected by the interaction between incubation time and mixing rate ($P=0.1364$) (Table 6). This is consistent with observations by Silva et al. (2018) who showed that extending the incubation period increased the yield of bioethanol, and beyond the optimum value the production capacity declined. The interactions of molasses concentration and incubation time with temperature were positively significant (Fig. 6c and d). Incubation time and temperature had the most significant positive impact on the response. Figure 6d demonstrates an interacting effect between molasses concentration and temperature that was positive and significant for bioethanol production ($P=0.0184$). The result showed an ethanol yield of 55.6% at 29.7 °brix and 31.9 °C. Subsequently, the ethanol yield began to decrease. The ethanol yield is enhanced until the optimum substrate concentration and temperature are reached. High substrate concentrations and temperatures cause osmotic shock and reduce enzyme activity, which has an inhibiting effect on the yeast cells (Azhar et al. 2017; Cavalaglio et al. 2016). According to Fig. 6e there is a direct correlation between the interaction of incubation time and temperature with the response ethanol yield, which was observed to be 55.1%. A positive interactive effect between incubation time and molasses concentration had a less significant impact on ethanol yield production is shown in Fig. 6f. As a result, with an initial molasses concentration of 30.9 °brix and 52.4 h fermentation duration, an ethanol yield of 55.5% was obtained. Finally, the model predicted as optimum fermentation conditions a molasses concentration of 30 °brix, mixing rate of 130 rpm, temperature of 30 °C, and an incubation time of 54 h resulting in a predicted maximum ethanol concentration of 29 $g.L^{-1}$ and an ethanol yield of 55.7%. The predicted model was experimentally verified through actual values of the bioethanol production that were obtained using a batch fermentation system resulting in a maximum of 35.5 $g.L^{-1}$ and 71% ethanol concentration and ethanol yield. Compared to the OVAT optimization, increments of 34% of ethanol concentration and ethanol yield were obtained. A lower value of bioethanol concentration of 18.6 $g.L^{-1}$ was reported by El-Mekkawi et al. (2019) using algae biomass of 98.7 g/L, inoculum volume of 15.09%, and incubation time of 43.6 h employing response surface methodology.

Figure 7a shows the predicted ethanol yield versus the observed yield and the data points are close to the regression line, indicating good agreement between predicted and experimentally obtained bioethanol yields ($g.L^{-1}$). The normal probability plot in the diagnostic plots showed that the residuals followed the normal probability distribution (Fig. 7b). The error distributions are approximately normal, and the model satisfies the assumptions of the analysis of variance (ANOVA) suggesting that the response data provided pertinent analysis. Overall, the current study has a limitation of accessing standards, specifically sugars to investigate the fermentation dynamics of glucose. Another drawback of this research is that the number of cells viable in each yeast isolate was not enumerated during the co-fermentation.

Limitations of the study

Scaling up the use of fermentative yeasts, either using a monoculture or coculture, from laboratory scale to pilot scale and industrial scale involves various uncertainties that need to be investigated to ensure the successful production of certain products such as bioethanol. This is especially true for the technological development of new yeast species and – strains. The first approach that needs to be considered is biological variability. The yeast strain under consideration may exhibit variations in growth rates, metabolic activity, and product yields under different conditions. This variability can impact the scalability of the fermentation process. In the case of our study, a co-fermentation of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-9, it was demonstrated that both individual and

(See figure on next page.)

Fig. 6 Response surface plots for optimization of independent fermentation variables to maximize bioethanol production of a co-fermentation of *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-19. Plots show the interaction between variables, viz. **a** temperature and mixing rate, (**b**) molasses concentration and mixing rate, (**c**) incubation time and mixing rate, (**d**) molasses concentration and temperature, (**e**) incubation time and temperature, and (**f**) incubation time and molasses concentration. Green, yellow, and red colors represent lower, medium, and higher levels of ethanol yield, respectively

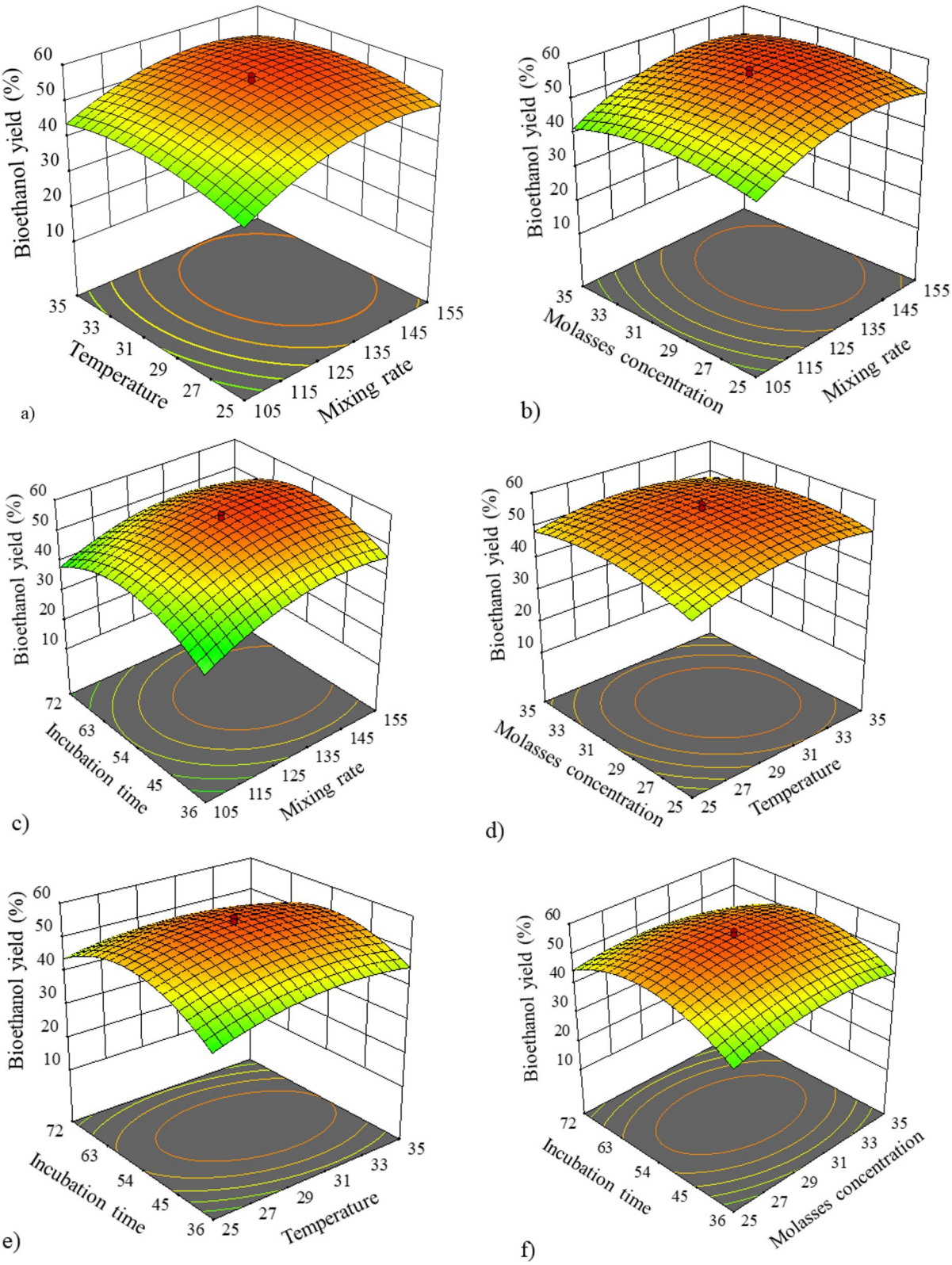

Fig. 6 (See legend on previous page.)

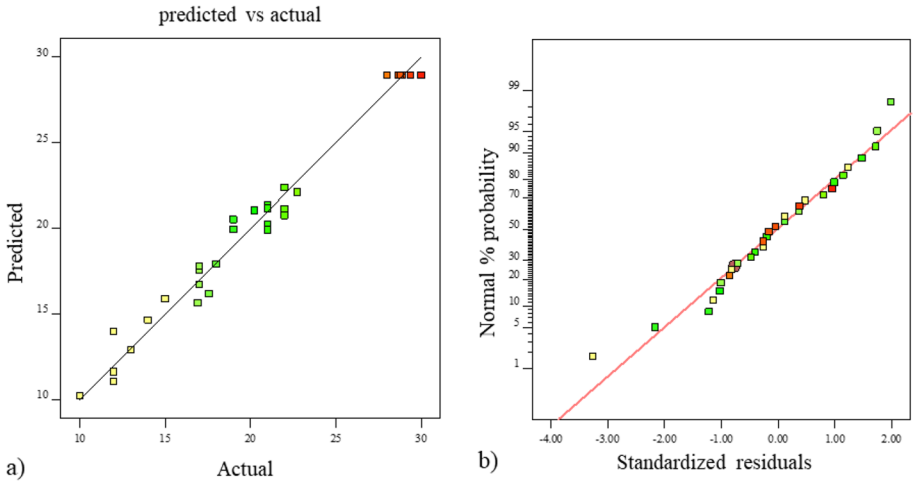

Fig. 7 Predicted versus actual values (**a**) and normal probability plot versus standardized residuals (**b**)

combined strains showed good stress tolerance characteristics and fermentation conditions under laboratory scale with high bioethanol production. However, the study of the fermentation kinetics of each isolate was not studied in the pilot scale.

Maintaining yeast viability and stability during large-scale fermentation and downstream processing is critical for consistent product quality. Factors such as ethanol concentration, oxidative stress, pH, and temperature fluctuations can affect yeast viability. *S. cerevisiae* TA2 and *W. anomalus* HCJ2F-9 showed viability under conditions relevant to industrial-scale ethanol production. This includes tolerance to high ethanol concentrations, temperature variations, pH fluctuations, and other stress factors commonly encountered in fermentation processes, but the performance of the strains at large scale needs to be assessed. Monitoring the genetic stability of the yeast isolates during repeated fermentation cycles was not investigated either. This is important to ensure consistent performance and avoid genetic drift, which can lead to undesired changes in fermentation characteristics and product quality over time.

The risk of contamination by undesirable microorganisms is another major factor that needs to be controlled. Contamination of the fermentation broth by other microbes can negatively impact product quality and yield. Microbial contaminants can have various impacts on yeast fermentation processes through nutrient competition, changes in pH, and the production of inhibitory compounds, e.g., antibiotics, toxins, mycotoxins, organic acids, phenolics, and volatile fatty acids. In the current study, the yeast isolates, both monoculture and coculture, demonstrated a potential to lower the pH of the fermentation medium in a short period into the acidic range, which could inhibit the growth of other microbes, including contaminants. However, the sensitivity of each yeast isolate against a specific inhibitory compound, e.g. mycotoxins produced by unwanted wild yeasts, was not investigated.

Finally, scaling up fermentation processes incurs significant capital and operating costs, including equipment procurement, facility modifications, utilities, and labor. Conducting thorough cost–benefit analyses and assessing the scalability of the process in terms of production costs, product quality, and market demand is essential for informed decision-making. This, of course, also depends on the developments of the global markets for fuels and biofuels.

Conclusion and future perspectives

The suitability of *S. cerevisiae* isolate TA2 and *W. anomalus* isolate HCJ2F-19 for bioethanol production, as indicated by good actual yields and productivity, suggests promising prospects for ethanol production. The result of this study showed that the co-fermentation of *S. cerevisiae* isolate TA2 and *W. anomalus* isolate HCJ2F-19 produced an ethanol yield of 71% under the optimum fermentation conditions obtained by response surface methodology based on central composite design (CCD). Adopting the mixed culture fermentation technique is relatively better compared to monoculture fermentation. The results showed that the growth of *W. anomalus* isolate HCJ2F-19 and *S. cerevisiae* isolate TA2 was not affected by each other during the process of co-fermentation. The mixed culture of *S. cerevisiae* isolate TA2 and *W. anomalus* isolate HCJ2F-19 produced a fermentation efficiency of 83.6% compared to the monocultures which gave values of 66.2% and 55.9%, respectively. The study's

findings indicate that fermenting sugarcane molasses with a mixed culture of less efficient ethanol producers (e.g. *W. anomalus* isolate HCJ2F-19 and *S. cerevisiae* isolate TA2) resulted in high amounts of ethanol. In conclusion, the utilization of a mixed culture comprising *W. anomalus* isolate HCJ2F-19 and *S. cerevisiae* isolate TA2 shows significant potential for enhancing bioethanol production from sugarcane molasses. The co-fermentation process leveraging the complementary attributes of these two yeast strains has been demonstrated to yield favorable outcomes, including improved ethanol yields and productivity. However, further research is required to comprehensively evaluate the response of these yeast isolates to various stress conditions during the propagation process and fermentation and elucidating how they alleviate or remedy them. This may involve studying yeast cells' gene expression patterns, metabolic pathways, and physiological changes under different stress conditions.

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Authors' contributions
The proposal's write-up and sample collection were carried out by Estifanos Hawaz, Mesfin Tafesse, Anteneh Tesfaye, Dereje Beyene, Solomon Kiros, Alene Admas, Ayantu Degefe, Sisay Degu, Gessese Kebede, and Diriba Muleta. Estifanos Hawaz analyzed the data for single-culture and mixed-culture fermentation kinetics and prepared the manuscript. Manuscript edition was carried out by Diriba Muleta and Teun Boekhout.

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Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
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References

Aditiya H, Mahlia T, Chong W, Nur H, Sebayang A (2016) Second generation bioethanol production: a critical review. Renew Sustain Energy Rev 66:631–653. https://doi.org/10.1016/j.rser.2016.07.015
Akalın MK, Karagöz S, Akyüz M (2013) Application of response surface methodology to extract yields from stinging nettle under supercritical ethanol conditions. J Supercrit Fluids 84:164–172. https://doi.org/10.1016/j.supflu.2013.10.004
Arshad M, Khan Z, Shah F, Rajoka M (2008) Optimization of process variables for minimization of byproduct formation during fermentation of blackstrap molasses to ethanol at industrial scale. Lett Appl Microbiol 47(5):410–414. https://doi.org/10.1111/j.1472-765X.2008.02446.x
Aswathy U, Sukumaran RK, Devi GL, Rajasree K, Singhania RR, Pandey A (2010) Bio-ethanol from water hyacinth biomass: an evaluation of enzymatic saccharification strategy. Biores Technol 101(3):925–930. https://doi.org/10.1016/j.biortech.2009.08.019
Azadi P, Malina R, Barrett SR, Kraft M (2017) The evolution of the biofuel science. Renew Sustain Energy Rev 76:1479–1484. https://doi.org/10.1016/j.rser.2016.11.181
Azhar SHM, Abdulla R, Jambo SA, Marbawi H, Gansau JA, Faik AAM, Rodrigues KF (2017) Yeasts in sustainable bioethanol production: a review. Biochem Biophys Rep 10:52–61. https://doi.org/10.1016/j.bbrep.2017.03.003
Balat M (2011) Production of bioethanol from lignocellulosic materials via the biochemical pathway: a review. Energy Convers Manage 52(2):858–875. https://doi.org/10.1016/j.enconman.2010.08.013
Balat M, Balat H (2009) Recent trends in global production and utilization of bio-ethanol fuel. Appl Energy 86(11):2273–2282. https://doi.org/10.1016/j.apenergy.2009.03.015
Basso LC, Basso TO, Rocha SN (2011) Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. Biofuel Production Recent Dev Prospects 1530:85–100
Bellido C, González-Benito G, Coca M, Lucas S, García-Cubero MT (2013) Influence of aeration on bioethanol production from ozonized wheat straw hydrolysates using Pichia stipitis. Biores Technol 133:51–58. https://doi.org/10.1016/j.biortech.2013.01.104
Boboye B, Dayo-Owoyemi I (2009) Evaluation of dough sensory properties impacted by yeasts isolated from cassava. J Appl Sci 9(4):771–776
Branco RH, Serafim LS, Xavier AM (2018) Second generation bioethanol production: on the use of pulp and paper industry wastes as feedstock. Fermentation 5(1):4. https://doi.org/10.3390/fermentation5010004
Braz A, Mateus MM, dos Santos RG, Machado R, Bordado JM, Correia MJN (2019) Modelling of pine wood sawdust thermochemical liquefaction. Biomass Bioenerg 120:200–210. https://doi.org/10.1016/j.biombioe.2018.11.001
Buijs NA, Siewers V, Nielsen J (2013) Advanced biofuel production by the yeast Saccharomyces cerevisiae. Curr Opin Chem Biol 17(3):480–488. https://doi.org/10.1016/j.cbpa.2013.03.036
Carrillo-Nieves D, Alanís MJR, de la Cruz Quiroz R, Ruiz HA, Iqbal HM, Parra-Saldívar R (2019) Current status and future trends of bioethanol production from agro-industrial wastes in Mexico. Renew Sustain Energy Rev 102:63–74. https://doi.org/10.1016/j.rser.2018.11.031
Cavalaglio G, Gelosia M, Ingles D, Pompili E, D'Antonio S, Cotana F (2016) Response surface methodology for the optimization of cellulosic ethanol production from Phragmites australis through pre-saccharification and simultaneous saccharification and fermentation. Ind Crops Prod 83:431–437. https://doi.org/10.1016/j.indcrop.2015.12.089
Chotineeranat S, Wansuksri R, Piyachomkwan K, Chatakanonda P, Weerathaworn P, Sriroth K (2010) Effect of calcium ions on ethanol production from molasses by Saccharomyces cerevisiae. Sugar Tech 12:120–124. https://doi.org/10.1007/s12355-010-0024-6
Choudhary J, Singh S, Nain L (2017) Bioprospecting thermotolerant ethanologenic yeasts for simultaneous saccharification and fermentation from diverse environments. J Biosci Bioeng 123(3):342–346. https://doi.org/10.1016/j.jbiosc.2016.10.007

Ciani M, Comitini F (2015) Yeast interactions in multi-starter wine fermentation. Curr Opin Food Sci 1:1–6. https://doi.org/10.1016/j.cofs.2014.07.001
Cronjé Y, Farzad S, Mandegari M, Görgens JF (2023) A critical review of multiple alternative pathways for the production of a high-value bioproduct from sugarcane mill byproducts: the case of adipic acid. Biofuel Res J 10(3):1933–1947. https://doi.org/10.18331/BRJ2023.10.3.5
De Vasconcelos J, Lopes C, De Franca F (2004) Continuous ethanol production using yeast immobilized on sugar-cane stalks. Braz J Chem Eng 21(3):357–365. https://doi.org/10.1590/S0104-66322004000300002
Demain AL, Newcomb M, Wu JD (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69(1):124–154
Demirbas A (2009) Political, economic and environmental impacts of biofuels: a review. Appl Energy 86:S108–S117. https://doi.org/10.1016/j.apenergy.2009.04.036
Dhyani V, Bhaskar T (2018) A comprehensive review on the pyrolysis of lignocellulosic biomass. Renew Energy 129:695–716. https://doi.org/10.1016/j.renene.2017.04.035
Dias MO, Cunha MP, Jesus CD, Rocha GJ, Pradella JGC, Rossell CE, Maciel Filho R, Bonomi A (2011) Second generation ethanol in Brazil: can it compete with electricity production? Biores Technol 102(19):8964–8971. https://doi.org/10.1016/j.biortech.2011.06.098
Dobrescu AC, Veras HCT, Varrone C, Knudsen JD (2021) Novel propagation strategy of saccharomyces cerevisiae for enhanced xylose metabolism during fermentation on softwood hydrolysate. Fermentation 7(4):288. https://doi.org/10.3390/fermentation7040288
Dodić S, Popov S, Dodić J, Ranković J, Zavargo Z, Mučibabić RJ (2009) Bioethanol production from thick juice as intermediate of sugar beet processing. Biomass Bioenerg 33(5):822–827. https://doi.org/10.1016/j.biombioe.2009.01.002
Domínguez-Bocanegra AR, Torres-Muñoz JA, López RA (2015) Production of bioethanol from agro-industrial wastes. Fuel 149:85–89. https://doi.org/10.1016/j.fuel.2014.09.062
Du Preez J, Bosch M, Prior B (1987) Temperature profiles of growth and ethanol tolerance of the xylose-fermenting yeasts Candida shehatae and Pichia stipitis. Appl Microbiol Biotechnol 25:521–525. https://doi.org/10.1007/BF00252010
Duarte WF, Dias DR, de Melo Pereira GV, Gervásio IM, Schwan RF (2009) Indigenous and inoculated yeast fermentation of gabiroba (Campomanesia pubescens) pulp for fruit wine production. J Ind Microbiol Biotechnol 36(4):557–569. https://doi.org/10.1007/s10295-009-0526-y
Dynesen J, Smits H, Olsson L, Nielsen J (1998) Carbon catabolite repression of invertase during batch cultivations of Saccharomyces cerevisiae: the role of glucose, fructose, and mannose. Appl Microbiol Biotechnol 50:579–582. https://doi.org/10.1007/s002530051338
El-Mekkawi SA, Abdo SM, Samhan FA, Ali GH (2019) Optimization of some fermentation conditions for bioethanol production from microalgae using response surface method. Bull Natl Res Centre 43:1–8. https://doi.org/10.1186/s42269-019-0205-8
Fadel M, Keera AA, Mouafi FE, Kahil T (2013) High level ethanol from sugar cane molasses by a new thermotolerant Saccharomyces cerevisiae strain in industrial scale. Biotechnol Res Int 2013. https://doi.org/10.1155/2013/253286
Farzad S, Mandegari MA, Guo M, Haigh KF, Shah N, Görgens JF (2017) Multi-product biorefineries from lignocelluloses: a pathway to revitalisation of the sugar industry? Biotechnol Biofuels 10:1–24. https://doi.org/10.1186/s13068-017-0761-9
Fathiah MF, Hartono FD, Astuti RI, Listiyowati S, Meryandini A (2024) Bioethanol Production from Non-Conventional Yeasts Wickerhamomyces anomalus (Pichia anomala) and Detection of ADH1 Gene. HAYATI J Biosci 31(2):221–228. https://doi.org/10.4308/hjb.31.2.221-228
Gebreegziabher Z, Mekonnen A, Ferede T, Kohlin G (2017) Profitability of bioethanol production: the case of Ethiopia. Ethiopian J Econ 26(1):101–122
Gutiérrez-Rivera B, Waliszewski-Kubiak K, Carvajal-Zarrabal O, Aguilar-Uscanga MG (2012) Conversion efficiency of glucose/xylose mixtures for ethanol production using Saccharomyces cerevisiae ITV01 and Pichia stipitis NRRL Y-7124. J Chem Technol Biotechnol 87(2):263–270. https://doi.org/10.1002/jctb.2709
Hawaz E, Tafesse M, Tesfaye A, Beyene D, Kiros S, Kebede G, Boekhout T, Theelen B, Groenewald M, Degefe A, Degu S, Admas A, Muleta D (2022) Isolation and characterization of bioethanol producing wild yeasts from bio-wastes and co-products of sugar factories. Ann Microbiol 72(1):39. https://doi.org/10.1186/s13213-022-01695-3
Hawaz E, Tafesse M, Tesfaye A, Kiros S, Beyene D, Kebede G, Boekhout T, Groenewald M, Theelen B, Degefe A (2023) Optimization of bioethanol production from sugarcane molasses by the response surface methodology using Meyerozyma caribbica isolate MJTm3. Ann Microbiol 73(1):2. https://doi.org/10.1186/s13213-022-01706-3
Heidari N, Pearce JM (2016) A review of greenhouse gas emission liabilities as the value of renewable energy for mitigating lawsuits for climate change related damages. Renew Sustain Energy Rev 55:899–908
Henderson CM, Lozada-Contreras M, Jiranek V, Longo ML, Block DE (2013) Ethanol production and maximum cell growth are highly correlated with membrane lipid composition during fermentation as determined by lipidomic analysis of 22 Saccharomyces cerevisiae strains. Appl Environ Microbiol 79(1):91–104. https://doi.org/10.1128/AEM.02670-12
Hill J, Nelson E, Tilman D, Polasky S, Tiffany D (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. Proc Natl Acad Sci 103(30):11206–11210. https://doi.org/10.1073/pnas.0604600103
Ho DP, Ngo HH, Guo W (2014) A mini review on renewable sources for biofuel. Biores Technol 169:742–749. https://doi.org/10.1016/j.biortech.2014.07.022
Hu N, Yuan B, Sun J, Wang S-A, Li F-L (2012) Thermotolerant Kluyveromyces marxianus and Saccharomyces cerevisiae strains representing potentials for bioethanol production from Jerusalem artichoke by consolidated bioprocessing. Appl Microbiol Biotechnol 95:1359–1368. https://doi.org/10.1007/s00253-012-4240-8
Jayanti A, Sutrisno A, Wardani A, Murdiyatmo U (2019) Bioethanol production from sugarcane molasses by instant dry yeast (effect of pretreatment and fermentation temperature). IOP Conference Series Earth Environ Sci. https://doi.org/10.1088/1755-1315/230/1/012102
Karagöz P, Özkan M (2014) Ethanol production from wheat straw by Saccharomyces cerevisiae and Scheffersomyces stipitis co-culture in batch and continuous system. Biores Technol 158:286–293. https://doi.org/10.1016/j.biortech.2014.02.022
Kasavi C, Finore I, Lama L, Nicolaus B, Oliver SG, Oner ET, Kirdar B (2012) Evaluation of industrial Saccharomyces cerevisiae strains for ethanol production from biomass. Biomass Bioenerg 45:230–238. https://doi.org/10.1016/j.biombioe.2012.06.013
Kurtzman CP, Robnett CJ, Basehoar-Powers E (2008) Phylogenetic relationships among species of Pichia, Issatchenkia and Williopsis determined from multigene sequence analysis, and the proposal of Barnettozyma gen. nov., Lindnera gen. nov. and Wickerhamomyces gen. nov. FEMS Yeast Res 8(6):939–954
Laopaiboon L, Nuanpeng S, Srinophakun P, Klanrit P, Laopaiboon P (2009) Ethanol production from sweet sorghum juice using very high gravity technology: effects of carbon and nitrogen supplementations. Biores Technol 100(18):4176–4182. https://doi.org/10.1016/j.biortech.2009.03.046
Lee P-R, Ong Y-L, Yu B, Curran P, Liu S-Q (2010) Profile of volatile compounds during papaya juice fermentation by a mixed culture of Saccharomyces cerevisiae and Williopsis saturnus. Food Microbiol 27(7):853–861. https://doi.org/10.1016/j.fm.2010.05.010
Lennartsson PR, Erlandsson P, Taherzadeh MJ (2014) Integration of the first and second generation bioethanol processes and the importance of by-products. Biores Technol 165:3–8. https://doi.org/10.1016/j.biortech.2014.01.127
Liang M, Kim MH, He QP, Wang J (2013) Impact of pseudo-continuous fermentation on the ethanol tolerance of Scheffersomyces stipitis. J Biosci Bioeng 116(3):319–326. https://doi.org/10.1016/j.jbiosc.2013.03.016
Liew WH, Hassim MH, Ng DK (2014) Review of evolution, technology and sustainability assessments of biofuel production. J Clean Prod 71:11–29. https://doi.org/10.1016/j.jclepro.2014.01.006

Lin Y, Zhang W, Li C, Sakakibara K, Tanaka S, Kong H (2012) Factors affecting ethanol fermentation using Saccharomyces cerevisiae BY4742. Biomass Bioenerg 47:395–401. https://doi.org/10.1016/j.biombioe.2012.09.019

Lin T-H, Guo G-L, Hwang W-S, Huang S-L (2016) The addition of hydrolyzed rice straw in xylose fermentation by Pichia stipitis to increase bioethanol production at the pilot-scale. Biomass Bioenerg 91:204–209. https://doi.org/10.1016/j.biombioe.2016.05.012

Malik H (2016) Utilization of agro-industrial wastes for the biomass production of baker's yeast M. Thesis, Punjab Agricultural University, Punjab

Martínez-Moreno R, Morales P, Gonzalez R, Mas A, Beltran G (2012) Biomass production and alcoholic fermentation performance of Saccharomyces cerevisiae as a function of nitrogen source. FEMS Yeast Res 12(4):477–485. https://doi.org/10.1111/j.1567-1364.2012.00802.x

Martinez-Pastor M, Marchler G, Schüller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J 15(9):2227–2235. https://doi.org/10.1002/j.1460-2075.1996.tb00576.x

Mathew AK, Crook M, Chaney K, Humphries AC (2014) Continuous bioethanol production from oilseed rape straw hydrosylate using immobilised Saccharomyces cerevisiae cells. Biores Technol 154:248–253. https://doi.org/10.1016/j.biortech.2013.12.044

Mendoza LM, de Nadra MCM, Farías ME (2007) Kinetics and metabolic behavior of a composite culture of Kloeckera apiculata and Saccharomyces cerevisiae wine related strains. Biotech Lett 29(7):1057–1063. https://doi.org/10.1007/s10529-007-9355-0

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428. https://doi.org/10.1021/ac60147a030

Mohapatra S, Dandapat SJ, Thatoi H (2017) Physicochemical characterization, modelling and optimization of ultrasono-assisted acid pretreatment of two Pennisetum sp. using Taguchi and artificial neural networking for enhanced delignification. J Environ Manage 187:537–549. https://doi.org/10.1016/j.jenvman.2016.09.060

Moonsamy TA, Mandegari M, Farzad S, Görgens JF (2022) A new insight into integrated first and second-generation bioethanol production from sugarcane. Ind Crops Prod 188:115675. https://doi.org/10.1016/j.indcrop.2022.115675

Morales P, Rojas V, Quirós M, Gonzalez R (2015) The impact of oxygen on the final alcohol content of wine fermented by a mixed starter culture. Appl Microbiol Biotechnol 99:3993–4003. https://doi.org/10.1007/s00253-014-6321-3

Moysés DN, Reis VCB, Almeida JRMd, Moraes LMPd, Torres FAG (2016) Xylose fermentation by Saccharomyces cerevisiae: challenges and prospects. Int J Mol Sci 17(3):207. https://doi.org/10.3390/ijms17030207

Mukasekuru MR, Kaneza P, Sun H, Sun FF, He J, Zheng P (2020) Fed-batch high-solids enzymatic saccharification of lignocellulosic substrates with a combination of additives and accessory enzymes. Ind Crops Prod 146:112156. https://doi.org/10.1016/j.indcrop.2020.112156

Mussatto SI, Dragone G, Guimarães PM, Silva JPA, Carneiro LM, Roberto IC, Vicente A, Domingues L, Teixeira JA (2010) Technological trends, global market, and challenges of bio-ethanol production. Biotechnol Adv 28(6):817–830. https://doi.org/10.1016/j.biotechadv.2010.07.001

Naito Y, Okai M, Ishida M, Takashio M, Urano N (2019) Bioethanol production from molasses by yeasts with stress-tolerance isolated from aquatic environments in Japan. Adv Microbiol 9(12):1000–1011. https://doi.org/10.4236/aim.2019.912065

Nigam PS, Singh A (2011) Production of liquid biofuels from renewable resources. Prog Energy Combust Sci 37(1):52–68. https://doi.org/10.1016/j.pecs.2010.01.003

Nissen P, Arneborg N (2003) Characterization of early deaths of non-Saccharomyces yeasts in mixed cultures with Saccharomyces cerevisiae. Arch Microbiol 180:257–263. https://doi.org/10.1007/s00203-003-0585-9

Pérez-Torrado R, Bruno-Bárcena JM, Matallana E (2005) Monitoring stress-related genes during the process of biomass propagation of Saccharomyces cerevisiae strains used for wine making. Appl Environ Microbiol 71(11):6831–6837. https://doi.org/10.1128/AEM.71.11.6831-6837.2005

Phisalaphong M, Srirattana N, Tanthapanichakoon W (2006) Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation. Biochem Eng J 28(1):36–43. https://doi.org/10.1016/j.bej.2005.08.039

Rahman SS, Hossain MM, Choudhury N (2013) Effect of various parameters on the growth and ethanol production by yeasts isolated from natural sources. Bangladesh J Microbiol 30(1–2):49–54

Raina N, Slathia PS, Sharma P (2020) Response surface methodology (RSM) for optimization of thermochemical pretreatment method and enzymatic hydrolysis of deodar sawdust (DS) for bioethanol production using separate hydrolysis and co-fermentation (SHCF). Biomass Conversion Biorefinery 1–21. https://doi.org/10.1007/s13399-020-00970-0

REN21 R (2017) Global status report, REN21 secretariat, Paris, France. Tech Rep 91–93

Robak K, Balcerek M (2018) Review of second generation bioethanol production from residual biomass. Food Technol Biotechnol 56(2):174. https://doi.org/10.17113/ftb.56.02.18.5428

Ruyters S, Mukherjee V, Verstrepen KJ, Thevelein JM, Willems KA, Lievens B (2015) Assessing the potential of wild yeasts for bioethanol production. J Ind Microbiol Biotechnol 42(1):39–48. https://doi.org/10.1007/s10295-014-1544-y

Satora P, Tarko T, Sroka P, Blaszczyk U (2014) The influence of Wickerhamomyces anomalus killer yeast on the fermentation and chemical composition of apple wines. FEMS Yeast Res 14(5):729–740. https://doi.org/10.1111/1567-1364.12159

Shi J, Zhang M, Zhang L, Wang P, Jiang L, Deng H (2014) Xylose-fermenting P ichia stipitis by genome shuffling for improved ethanol production. Microb Biotechnol 7(2):90–99. https://doi.org/10.1111/1751-7915.12092

Silva C, Meneghello D, Bertucco A (2018) A systematic study regarding hydrolysis and ethanol fermentation from microalgal biomass. Biocatal Agric Biotechnol 14:172–182. https://doi.org/10.1016/j.bcab.2018.02.016

Singh A, Bajar S, Bishnoi NR (2014) Enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by Saccharomyces cerevisiae, Scheffersomyces stipitis and their co-culture. Fuel 116:699–702. https://doi.org/10.1016/j.fuel.2013.08.072

Song Y, Cho EJ, Park CS, Oh CH, Park B-J, Bae H-J (2019) A strategy for sequential fermentation by Saccharomyces cerevisiae and Pichia stipitis in bioethanol production from hardwoods. Renew Energy 139:1281–1289. https://doi.org/10.1016/j.renene.2019.03.032

Thompson W, Meyer S (2013) Second generation biofuels and food crops: co-products or competitors? Glob Food Sec 2(2):89–96. https://doi.org/10.1016/j.gfs.2013.03.001

Turner W, Greetham D, Du C (2022) The characterisation of Wickerhamomyces anomalus M15, a highly tolerant yeast for bioethanol production using seaweed derived medium. Front Bioengineer Biotechnol 10:1028185. https://doi.org/10.3389/fbioe.2022.1028185

Van Maris AJ, Winkler AA, Kuyper M, De Laat WT, Van Dijken JP, Pronk JT (2007) Development of efficient xylose fermentation in Saccharomyces cerevisiae: xylose isomerase as a key component. Biofuels, 179–204. https://doi.org/10.1007/10_2007_057

Vučurović DG, Dodić SN, Popov SD, Dodić JM, Grahovac JA (2012) Process model and economic analysis of ethanol production from sugar beet raw juice as part of the cleaner production concept. Biores Technol 104:367–372. https://doi.org/10.1016/j.biortech.2011.10.085

Wu Y, Wen J, Wang K, Su C, Chen C, Cui Z, Cai D, Cheng S, Cao H, Qin P (2023) Understanding the dynamics of the saccharomyces cerevisiae and scheffersomyces stipitis abundance in co-culturing process for bioethanol production from corn stover. Waste Biomass Valorization 14(1):43–55. https://doi.org/10.1007/s12649-022-01861-3

Yadav KS, Naseeruddin S, Prashanthi GS, Sateesh L, Rao LV (2011) Bioethanol fermentation of concentrated rice straw hydrolysate using co-culture of Saccharomyces cerevisiae and Pichia stipitis. Biores Technol 102(11):6473–6478. https://doi.org/10.1016/j.biortech.2011.03.019

Ye M, Yue T, Yuan Y (2014) Effects of sequential mixed cultures of Wickerhamomyces anomalus and Saccharomyces cerevisiae on apple cider fermentation. FEMS Yeast Res 14(6):873–882. https://doi.org/10.1111/1567-1364.12175

Yücel HG, Aksu Z (2015) Ethanol fermentation characteristics of Pichia stipitis yeast from sugar beet pulp hydrolysate: use of new detoxification methods. Fuel 158:793–799. https://doi.org/10.1016/j.fuel.2015.06.016

Zha Y, Hossain AH, Tobola F, Sedee N, Havekes M, Punt PJ (2013) Pichia anomala 29X: a resistant strain for lignocellulosic biomass hydrolysate fermentation. FEMS Yeast Res 13(7):609–617. https://doi.org/10.1111/1567-1364.12062

Zhang K, Wells P, Liang Y, Love J, Parker DA, Botella C (2019) Effect of diluted hydrolysate as yeast propagation medium on ethanol production. Biores Technol 271:1–8. https://doi.org/10.1016/j.biortech.2018.09.080
Zhou H, Cheng J-S, Wang BL, Fink GR, Stephanopoulos G (2012) Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by Saccharomyces cerevisiae. Metab Eng 14(6):611–622. https://doi.org/10.1016/j.ymben.2012.07.011
Zohre D, Erten H (2002) The influence of Kloeckera apiculata and Candida pulcherrima yeasts on wine fermentation. Process Biochem 38(3):319–324. https://doi.org/10.1016/S0032-9592(02)00086-9

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