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Bioethanol Production by Consolidated Bioprocessing and Simultaneous Saccharification and Fermentation of Sorghum Flour Using *Pichia* and *Meyerozyma guilliermondii* Strains

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Abstract

The urgent need for renewable energy sources worldwide has heightened due to worries about climate change, energy security, and the limited supply of fossil fuels, necessitating the need for other sustainable substitute such as bioethanol. This study is aimed at applying consolidated bioprocessing (CBP) and simultaneous saccharification and fermentation (SSF) processes for bioethanol production from sorghum flour using *Pichia* and *Meyerozyma guilliermondii* strains. Sorghum grains, purchased from a local market were ground to flour using an industrial grinding machine, while *Pichia* strains were obtained from a stock culture and *M. guilliermondii*, isolated from fresh palm wine and characterised. Also, about 1.6kg of corn grains were subjected to malting to produce alpha-amylase. The obtained amylase was partially purified using 80 % ammonium sulphate saturation followed by dialysis yielding a specific activity of 23.22 U/mg and total protein of 0.9098 mg/ml. The partially purified enzyme upon characterization was found to act optimally at a pH of 5.0, temperature of 50°C with a *K_m* and *V_{max}* values of 0.0024 mM⁻¹ and 2400 U⁻¹ respectively. The obtained enzyme was further employed in CBP and SSF for bioethanol production. The fermentation was carried out for 7 days and fermentation parameters were estimated using appropriate formula. The results showed that SSF of *Meyerozyma guilliermondii* exhibited maximum bioethanol yield of 0.274 g/g with fermentation efficiency of 53.62% after 4 days of fermentation, while CBP exhibited 0.177 g/g of bioethanol with fermentation efficiency of 34.64% after 2 days. CBP and SSF of *Pichia* strain Y exhibited 0.192 and 0.135 g/g bioethanol yield with a 37.57% and 26.42% fermentation efficiency after a day respectively, while CBP of *Pichia* strain Z exhibited 0.142 g/g bioethanol yield with 27.79% fermentation efficiency after 2 days, and SSF exhibited 0.140 g/g bioethanol with fermentation efficiency of 27.40% after a day. From the results, *M. guilliermondii* efficiently utilised sorghum flour for bioethanol production and demonstrated more tolerance to elevated levels of glucose and ethanol when compared to *Pichia* strains. Effective utilisation of these optimised conditions could play significant roles in efficient use of these organism for industrial production of bioethanol.

Keywords: Consolidated Bioprocessing, Simultaneous Saccharification Fermentation, Sorghum flour, *Meyerozyma guilliermondii*, *Pichia*, Bioethanol.

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INTRODUCTION

The global reliance on fossil fuels as source of energy has brought about considerable damages to the environment such as greenhouse gas emissions and climate change, making it necessary to transition to sustainable and renewable energy sources through environmentally friendly processes (Antunes et al., 2023). Bioethanol is an alcohol that is produced by the microbial fermentation reaction of carbohydrates and utilizing various types of sugar-rich substrates such as sugar cane, corn, sorghum etc could deliver efficient fermentation reactions (Fathiah et al., 2024). During the fermentation process, microorganisms break down organic molecules into simpler products, including ethanol, carbon dioxide, and other by-products. Currently, the focus of bioethanol production centres on its application within the transportation sector (Yaverino-Gutiérrez et al., 2024). Based on the substrate used for the fermentation, Bioethanol is classified into 1st generation, 2nd Generation, 3rd Generation and 4th Generation. Feedstocks high in sucrose (such as sugar cane, sugar beet, sweet sorghum, and fruits) and starch (corn, wheat, rice, potato, cassava, sweet potato, and barley) are used in the production of first-generation bioethanol (Devi et al., 2023). Wood, straw, grasses, and food industrial wastes are examples of lignocellulosic biomass used to produce second-generation bioethanol (Coulibaly et al., 2023). Algal biomass, which includes both microalgae and macroalgae, has been used to produce third-generation bioethanol while the fourth-generation is an extension of the third-generation that involves the production of genetically engineered algae or microorganisms for more biomass to augment biofuel production (Chandrasekhar et al., 2023). Sorghum (*Sorghum bicolor*) belongs to the Andropogoneae tribe and *Poaceae* family and is a known C4 crop (i.e., it uses the C4 carbon fixation pathway to increase its photosynthetic efficiency), particularly adapted to hot, drought-prone, and semi-arid tropical environments with less rainfall (Li et al., 2022). It's starch, protein, fat, and fibre content are between 55.60–76.20%, 4.4–14.86%, 1.38–10.54% and 1–7.94% respectively, depending on the cultivar (Dabija et al., 2021). In addition, several features make sorghum one of the most interesting cereal crops, including resistance to extreme agronomic conditions, resilience, low input requirements and high production yield (Hamadou et al., 2023). Consolidated bioprocessing (CBP) and simultaneous saccharification and fermentation (SSF) have emerged as promising strategies for bioethanol production, offering potential advantages in terms of cost-effectiveness, energy efficiency, and reduced processing steps compared to traditional methods and offering new avenues for improving ethanol yields, substrate utilisation, and overall process sustainability (Banner et al., 2021; Manikandan et al., 2022). CBP involves the integration of enzyme production, substrate hydrolysis, and fermentation into a single step, thereby simplifying the overall process and reducing the need for costly enzyme additions CBP systems streamline bioethanol production by reducing the need for

separate enzymatic hydrolysis and fermentation steps, thus minimizing operational costs, and simplifying process integration. (Ramos et al., 2023). Simultaneous saccharification and fermentation (SSF) is another innovative fermentation strategy wherein enzymatic hydrolysis of biomass and fermentation of sugars occur concurrently in a single vessel. SSF systems offer advantages in terms of reduced processing time, improved sugar utilisation, and enhanced ethanol yields compared to traditional separate hydrolysis and fermentation (SHF) processes (Derman et al., 2022). The optimization of microbial dynamics in CBP and SSF processes becomes crucial and microbial strains, particularly yeasts like *Pichia*, *Meyerozyma*, *Wickerhamomyces*, and *Lodderomyces* strains play a central role in fermentative bioconversion, due to their robust fermentation characteristics, versatility in utilising various sugars, and compatibility with the CBP and SSF framework (Ahuja et al., 2023). The selection and tailored application of these strains to specific grain substrates are essential for maximizing bioethanol yields and ensuring economic viability (Li et al., 2022).

Materials and Methods

Collection of samples

Sorghum grains were purchased from Ogige market, Nsukka, Enugu State, Nigeria, and identified by Mr Alfred Ozioko of the Department of Plant Science and Biotechnology, University of Nigeria Nsukka. 1kg of it was ground to obtain sorghum flour using an industrial grinding machine, while 1.6kg were malted for α -amylase production.

Collection of microorganism

Two *Pichia* strains were obtained from a stock culture of the Department of Biochemistry, University of Nigeria Nsukka, while *Meyerozyma guilliermondii* strain Q2 was isolated from fresh palm wine and characterised.

Chemicals and Reagents

All chemicals/reagents utilised in this study were of analytical grade and were obtained from Sigma-Aldrich (U.S.A.).

Steeping of the sorghum grains

1620.60g of Sorghum was weighed and washed using running tap water to remove dirt and unwanted particles. This was later poured into a 2000ml beaker containing 1000ml of water and was allowed to stand for 24hrs under room temperature.

Determination of the effects of malting period on α -amylase

The effect of malting period on α -Amylase was carried out by weighing 200g of Sorghum into seven different plates. These were evenly spread and allowed to germinate under

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room temperature for seven days. The plates were watered three times daily to maintain moist environment.

Determination of malt yield

Malt yield for each day of the malting period on percentage dry basis was determined by taking the weights of an equal gram of the grain before and after malting. This is expressed below:

$$\text{Malt yield (\%)} = \frac{\text{wt of grain before malting}}{\text{wt of grain after malting}} \times 100 \dots \dots \dots (1)$$

Determination of malt loss

Malt loss indicates the material loss expressed as a percentage of dry weight in converting the Sorghum grains to malt. The malt loss for each day of the malting period was evaluated by noting the difference in the dry weights of sorghum before and after malting. The difference was further divided by the dry weight of sorghum grains before malting and expressed as a percentage.

$$\text{Malt Loss (\%)} = \frac{\text{wt of grain before malting} - \text{wt of grain after malting}}{\text{wt of grain before malting}} \times 100 \dots (2)$$

Effect of malting period on α -amylase from sorghum

Sorghum Grains' α -Amylase was extracted by weighing 50g of the malted grain for each day. This was followed by blending it in 200ml of Acetate buffer of pH 5.5 until a

$$\text{Activity (U/mL)} = \frac{(\Delta A_{540\text{nm}} / \text{minute}) \times \text{Total volume reaction mixture (mL)} \times \text{dilution factor}}{\text{Molar extinction coefficient of glucose} \times \text{volume of enzyme used} \times \text{1cm path-length}} \dots \dots \dots (3)$$

Protein determination

The protein content was estimated as described by Lowry et al. (1951) using different concentrations of bovine serum albumin (BSA) (2 mg/mL) for the standard curve.

Purification of α -amylase

Ammonium sulphate precipitation

Ammonium sulphate profile of α -Amylase obtained from malted Sorghum Grains was carried out using different weight of ammonium salt with equivalent percentages of 20 % (0.53 g), 30 % (0.82 g), 40 % (1.10 g), 50 % (1.46 g), 60 % (1.81 g), 70 % (2.18 g), 80 % (2.58 g), 90 % (3.02 g) and finally 100 % (3.46 g) saturation as described by Chilaka et al. (2002). These equivalent masses of the salt were weighed respectively into 5 mL of the enzyme(s) in a 20 mL capacity tube. The suspension was stirred using a glass rod and kept in the fridge (4 °C) for 24 hrs. The solution was centrifuged at 4,000 rpm for 20 min to separate the supernatant and the pellet. Activity assays were carried out and eighty percent (80 %) ammonium sulphate saturation was suitable for concentration of the protein.

homogenate is formed. The homogenate was filtered using a four-fold muslin cloth and centrifuged at 5000rpm for 15mins. The supernatant obtained (crude α -Amylase) was put in a storage bottle and kept in a refrigerator following enzyme assay and protein determination. This was repeated until the 7th day of malting followed by mass production of the enzyme using the optimal day of enzyme production.

Assay for α -amylase activity using 3, 5-dinitrosalicylic acid (DNSA) method.

The level of α -amylase produced on each day of the malting period was inferred from the amount of reducing sugars produced upon its reaction on soluble starch. Alpha-amylase activity was assayed by estimating the release of glucose from starch using 3, 5-dinitrosalicylic acid (DNSA) method by Miller (1959) as illustrated in Wang et al. (1997). This was performed by adding 0.5ml of 2% starch, 0.5ml of 0.1M acetate buffer (pH 6.0), 0.5 ml of the crude enzyme into a test tube. This was incubated at 50°C for 30minutes after which 1ml of DNSA reagent was added, mixed thoroughly, and boiled for 10minutes. 1ml of Sodium-Potassium-Tartrate (Na⁺-K⁺-Tartrate) (Rochelle) solution and 1ml of distilled water were then added and allowed to cool. The absorbance was read at 540nm. A unit of enzyme activity however was described as the quantity of enzyme which catalyzes the release of one micromole of glucose per min using glucose standard curve.

Dialysis

The precipitated sorghum α -Amylase was dialyzed against 0.1 M acetate buffer, pH 5.5 for 6 hrs. according to the method described by Chilaka et al. (2002). An aliquot (10 mL) of the protein was poured into the dialysis membranes. The dialysis membranes or bags were tightened end-to-end with a string extending 10 cm at both ends. The membranes with the protein contents were immersed into the acetate buffer in an ice bath. The content of the membrane (dialyzed sorghum α -Amylase) was collected and stored in a refrigerator for α -Amylase assay and protein determination.

Characterization of partially purified α -amylase

Effect of pH on α -amylase activity

The pH studies on the α -Amylase activity were carried out as described by Simpson et al. (2006). Different buffer solutions, 0.1 M sodium acetate buffer (pH 3.0-5.5), 0.1 M sodium phosphate buffer (pH 6.0-7.5) and 0.1 M Tris-HCl buffer (pH 8.0-8.5) at 0.5 intervals were prepared by multiplying the concentration with the molecular weight of each salt to obtain the mass concentration (in g/L).

Effect of temperature on α -amylase activity

The effect of temperature on α -Amylase activity was determined at different temperature ranges (40-90 °C) at 10 °C interval using a temperature-controlled water-bath at optimum pH, as described by Simpson et al. (2006). The enzyme activity was determined as described earlier.

Effect of substrate concentration on α -amylase activity

The effect of different substrate concentrations on α -Amylase was determined as described by Sandalli et al. (2014) using glucose solutions of various concentration (0.5 – 10 % w/v) at pH 5. Activity assay was performed at optimum conditions of pH and temperature. The maximum velocity (V_{max}) and the Michaelis constant (K_m) were obtained from Lineweaver-Burk plot of initial velocity data.

Isolation of Fungal isolates

Preparation of culture medium

Potato Dextrose Agar (PDA) a non-selective media for growing fungi was used to isolate the *Meyerozyma guilliermondii* strain Q2 from fresh palm wine. Potato dextrose agar (3.9 % w/v) was prepared by dissolving 3.9g of PDA in 100 mL of distilled water in a conical flask. It was sterilised by autoclaving at 121 °C, for 15 minutes after which, the liquid PDA medium (45-50 °C) was poured into sterile petri-dishes and allowed to gel at room temperature for 1hr before inoculation by swabbing of palm wine using sterile cotton bud. The fungi were then allowed to grow for at least 48 hours before the subculturing process.

Sub-culturing of fungal growth

The organism grown from 2.2.10, together with two (2) *Pichia* strains (*Pichia* strains Y and Z) gotten from a stock culture, were sub-cultured several times to obtain pure isolates which were further characterized and employed in CBP and SSF processes for bioethanol production. The extraction of fungal genome was carried out using ZR Fungal/Bacterial DNA Miniprep extraction kit (by Zymo Research). Internal Transcribed Spacer (ITS) gene amplification of the fungal isolate was done using forward (ITS 1: TCC GTA GGT GAA CCT GCG G) and reverse primer (ITS 4: TCCTCCGCTTATTGATATGS). The Cycling Conditions for the Internal Transcribed Spacer (ITS) gene were set as follows; Initial denaturation at 94 °C for 5mins, followed by 36 cycles of denaturation at 94 °C for 30sec, annealing at 50 °C for 30secs and elongation at 72 °C for 45sec. Followed by a final elongation step at 72 °C for 7 minutes and hold temperature at 10 °C forever. The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Evolutionary relationship of organism was done using the UPGMA method. Maximum likelihood bootstrap method was utilized in computing the evolutionary distances while MEGA11

software was used for evolutionary tree drawing (Tamura et al., 2021).

Strain and inoculum preparation for bioethanol production

Five (5) disks each of the yeasts (*Pichia* strain Y, *Pichia* strain Z and *Meyerozyma guilliermondii* strain Q2) cultures were prepared in a Potato Dextrose Agar (PDA) media, then, they were used as the inoculum for the bioethanol production studies. The inoculum was inoculated into a 250 mL Erlenmeyer conical flask containing 100 mL of the fermentation media.

Submerged fermentation

Consolidated bioprocessing for fermentation of sorghum flour

The fermentation of grain flour was carried out in line with the methods of Channipa et al. (2018). The process was carried out in sterile 250 mL Erlenmeyer flasks with a total working volume of 100 mL containing 20% grain flour, 0.6% KH_2PO_4 , 0.5% $(NH_4)_2SO_4$, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.01% $FeSO_4$ nutrient solution. The flasks were covered with an aluminium foil, autoclaved at 121 °C for 15 minutes, and then, allowed to cool at room temperature. After cooling, 5 disks of microbial strains (*Pichia* strain Y, *Pichia* strain Z and *Meyerozyma guilliermondii* strain Q2) were added to each fermentation flask and incubated at room temperature for 7 days. During the fermentation period, samples were taken from the fermentation broth every day to determine the bioethanol concentration and all experiments were performed in duplicate.

Simultaneous saccharification and fermentation

The SSF media (100 mL) was made up of the same fermentation media stated in 2.2.11.1 above, followed by the addition of 2mL of partially purified alpha-amylase obtained from malted sorghum grains before incubation at room temperature for 7 days.

Determination of reducing sugars

The quantity of reducing sugars in the fermented broth was assayed using the Dinitrosalicylic acid (DNSA) method of Miller, (1959). The concentration of the reducing sugar in the fermented broth was estimated by the addition of 1 mL of 3,5-dinitrosalicylic acid to 1 mL of each of the fermented samples and boiling for 5 minutes, followed by the addition of 1 mL distilled water. The reagent blank was prepared by the addition of 1 mL of distilled water and 1 mL of DNSA reagent and boiling for 5 minutes, followed by the addition of 1 mL of distilled water. After this, 1 mL of 40% Rochelle salt solution was added to both mixtures and then allowed to cool. The absorbance was read at 540 nm using a spectrophotometer and the concentration of reducing sugars was estimated from the glucose standard curve.

Determination of bioethanol produced

The concentration of bioethanol, produced from grain flour, was measured using the method described by Oyeleke & Jibrin, (2009) and Caputi et al. (1968). 0.5 mL of the fermented biomass was put into test tubes, and 7 mL of acidified chromium reagent (concentrated potassium dichromate) was then added into the test tubes. This was allowed to stand for 30 minutes for colour development. The absorbance of each ethanol concentration was read at 600 nm using a spectrophotometer. The Bioethanol content of each sample was estimated from the ethanol standard curve.

Determination of bioethanol yield and productivity

The bioethanol yield and productivity of the grain flours using *Pichia* strain Y, *Pichia* strain Z, and *Meyerozyma guilliermondii* strain Q2 in CBP and SSF were achieved using the following parameters: sugar utilisation, ethanol yield, ethanol productivity and fermentation efficiency.

Determination of sugar utilisation

This was calculated using the following formula.

$$\text{Sugar Utilisation (\%)} = \frac{\text{Original sugar content} - \text{Residual sugar content}}{\text{Original sugar content}} \times 100 \dots\dots\dots(4)$$

Determination of bioethanol yield

This was calculated using the following formula.

$$\text{Bioethanol yield (g/g)} = \frac{\text{Maximum bioethanol concentration (g/L)}}{\text{Utilized glucose (g/L)}} \dots\dots\dots(5)$$

Determination of bioethanol productivity

This was calculated using the following formula.

$$\text{Bioethanol Productivity (g|L|h)} = \frac{\text{Maximum bioethanol concentration (g/L)}}{\text{Fermentation period (h)}} \dots\dots\dots(6)$$

Determination of fermentation efficiency

This was calculated using the following formula.

$$\text{Fermentation efficiency (\%)} = \frac{\text{Actual bioethanol yield (g/L)}}{\text{Theoretical yield of bioethanol (g/L)}} \times 100 \dots\dots\dots(7)$$

Results and Discussion

Determination of malting parameters (malt yield and malt loss)

The increase in malt loss and decrease in malt yield up to the point where they meet after day 5 signifies a point highest activity of the enzymes as well as the breakdown of the starch molecule which indicates highest germination (Figure 1). This is in line with Ofoedu et al. (2022) in their article on “Original article Effect of malting regimen on diastatic power, cold and hot water extracts of malts from sorghum”, which opines that as steeping increases water availability, grain softening and stimulation of grain to germinate, germination, on the other hand, promotes the production of hydrolytic

enzymes for grain transformation. This balance suggests that day 5 represents the ideal malting period for obtaining maximum alpha amylase activity while maintaining an acceptable malt yield.

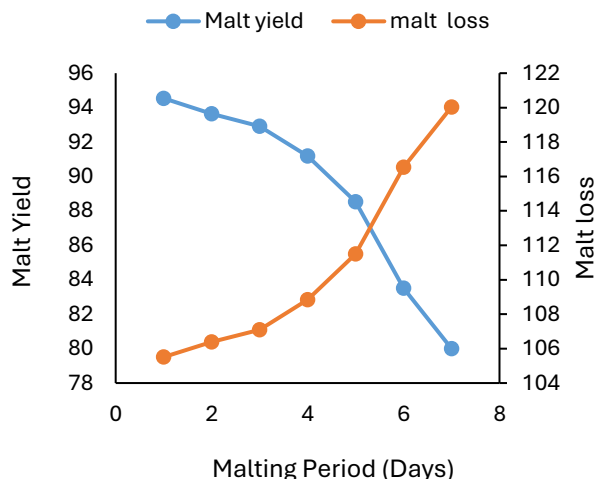


Figure 1: Effect of malting period on malting parameters of sorghum

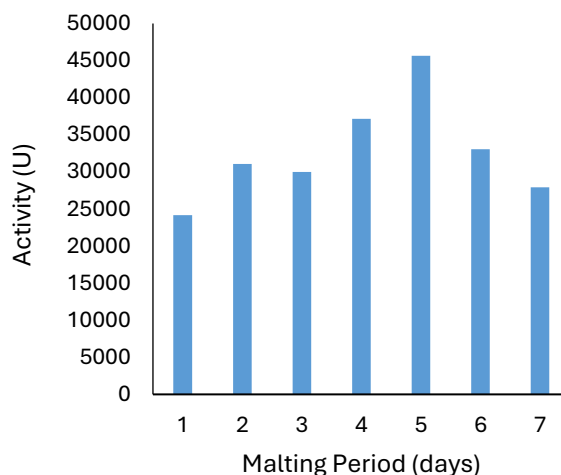


Figure 2: Effect of malting period on alpha-amylase activity obtained from sorghum.

Effect of malting period on alpha amylase

During the malting process of sorghum, it was observed that the optimum activity of alpha amylase occurred on day 5 of the 7-day malting period (Figure 2). This finding suggests that specific conditions in the malting process are conducive to enzyme activity, which peaked on day 5. This observation aligns with previous research indicating that the malting can significantly impact enzyme activity. According to (Kawarygielska & Opiela, 2023), Sorghum malts germinated for 24, 48 and 72 h were characterized with poor technological parameters, but 96 and 120 h germination period allowed for the production of malts which could be used in the beer

brewing process. This confirms that malting period affect the activity and quality of alpha amylase extract of the malt.

Partial purification of α -amylase obtained from sorghum Ammonium sulphate precipitation profile

The ammonium sulphate precipitation profile of the obtained alpha-amylase attained optimum saturation at 80% (Figure 3). This showed that 80% of ammonium sulphate was adequate to precipitate maximum amount of the enzyme with a specific activity of 11.29 (U/mg), which was found to increase upon dialysis to 23.22 (U/mg) (Table 1).

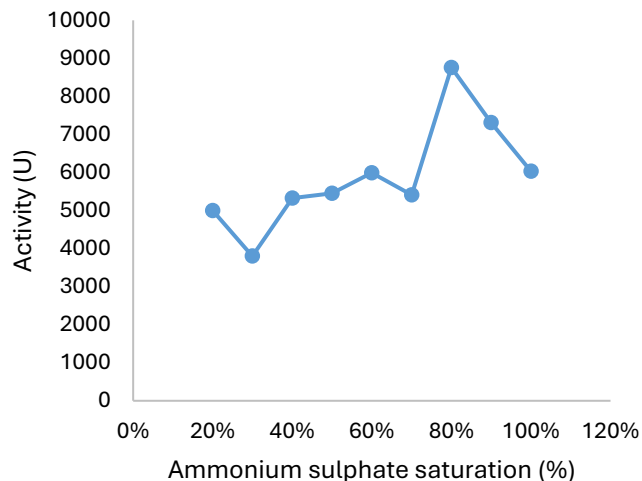


Figure 3: Ammonium sulphate precipitation of α -amylase from sorghum

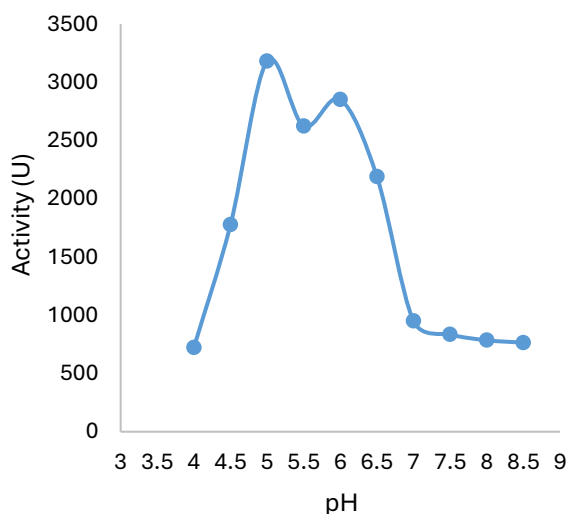


Table 1: Alpha-amylase purification profile

Purification steps	Total Protein (mg)	Total activity (U)	Specific Activity (U/mg)	Purification Fold	% Yield
Crude	5163.16	33057.85	6.40	1.0	100
Ammonium sulphate precipitate	38.91	440.12	11.29	1.76	1.33
Dialysed	0.91	21.13	23.22	2.06	4.80

Characterization of α -amylase

Effect of pH and temperature on α -amylase

The result of the effect of varying pH and temperature on the activity of alpha-amylase obtained from sorghum, showed an optimum pH of 5.0 and temperature of 50°C (Figure 4). These conditions represent the ideal range for utilizing this enzyme in specific processes, such as brewing or starch hydrolysis.

Effect of substrate concentration

From the double reciprocal plot of initial velocity data at varying concentrations, the Michaelis-Menten constant (K_m) was found to be 0.0024 (mM^{-1}) and the maximum velocity (V_{max}), 2400 (U^{-1}) (Figure 5). The calculated K_m and V_{max} provide quantitative insights into the enzyme's substrate affinity and catalytic efficiency and are consistent with the enzymatic characteristics reported by Adewale et al. (2006), further confirming the substrate affinity and catalytic efficiency of alpha amylase from sorghum. These values can aid in the design and optimization of enzymatic processes involving alpha amylase.

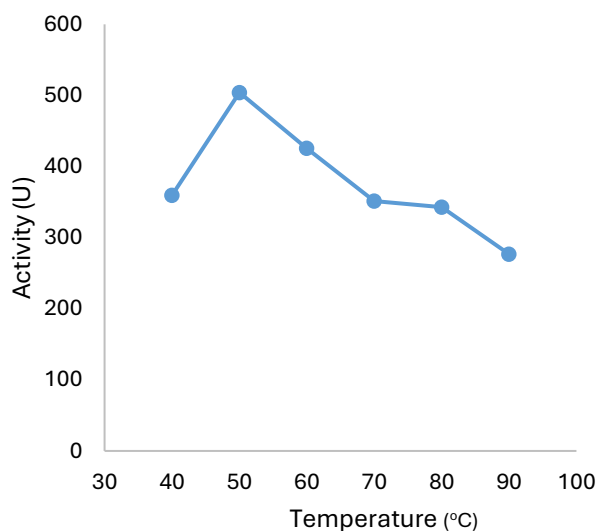


Figure 4: Effect of (A) pH on α -amylase activity, (B) temperature on α -amylase activity

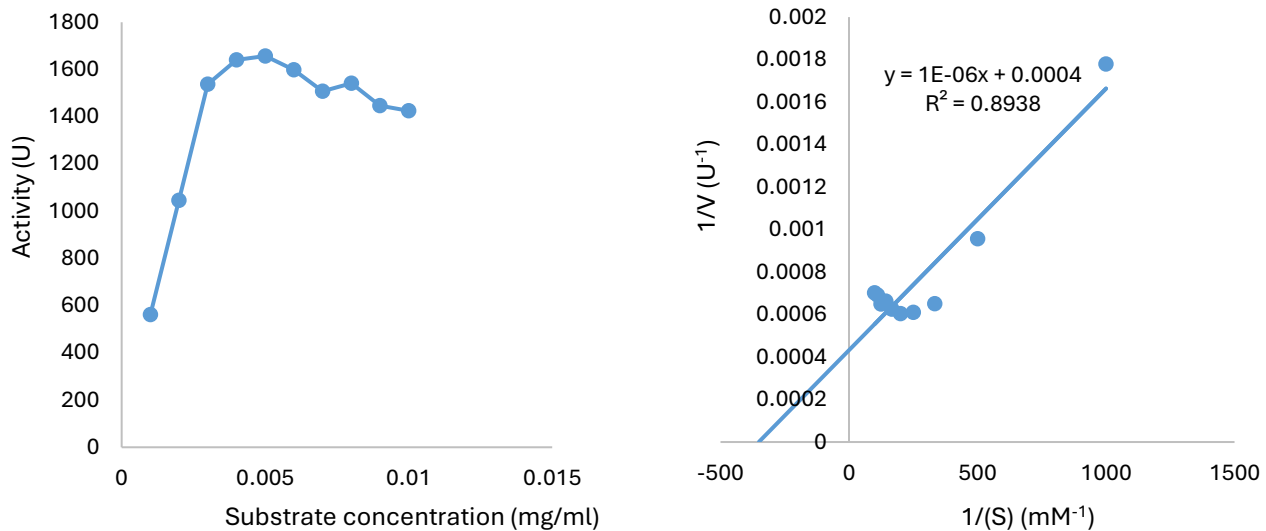


Figure 5: (A) Effect of substrate concentration on α -amylase activity (B) Double reciprocal plot

3.5 Isolation and molecular identification of *Meyerozyma guilliermondii* Q2

The agarose gel electrophoresis result showed a band at 650 bp (Figure 6) which is indicative of *Meyerozyma guilliermondii* genus (Grijalva-Vallejos et al., 2020). The NCBI blast query sequence analyses and phylogenetic history further revealed the similarity of the fungal strain to *Meyerozyma guilliermondii* Q2 (Figure 7), having a bootstrap value of 98.09 % and accession number of **MG601177**. The bootstrap score of 98.09% reveals very high similarity between *Meyerozyma guilliermondii* Q2 (Target) with *Meyerozyma guilliermondii* strain OTU70 from the NCBI GenBank.

Genetic sequence of *Meyerozyma guilliermondii* Q2

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CATCTACTCCTAAAAAAGGATCTTCTGCAGCG
CTTAAGTGCAGCGGCGAAAAACCTTACACACAGTG
TCTTTTTGATAAAGAACTCTTGATTGGTTGGCC
TAGAGATAGGTTGGGCCAGAGGTTTAAACAAAAC
ACAATTTAATTATTTTACAGTTAGTCAAATTTTG
AATTAATCTTCAAAACTTTCAACAACGGATCTCT
TGGTCTCGCATCGATGAAGAACGCAGCGAAATG
CGATAAGTAATATGAATTGCAGATTTTCGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCTCTGG
TATTCCAGAGGGCATGCCTGTTTGAGCGTCATTT
CTCTCTCAAACCCCGGGTTTGGTATTGAGTGAT
ACTCTTAGTCGGACTAGGCGTTTGCTTGAAAAGT
ATTGGCATGGGTAGTACTAGATAGTGCTGTGCAC
CTCTCAATGTATTAGTTTATCCAACCTCGTTGAAT
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GGTGTGGCGGGAGATTTGTGGTATTGTTGGCCCG
GCCTTACAACAACCAACAAGTTTGACCTCAAAT
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CCATAAAGCGGAGAGAA
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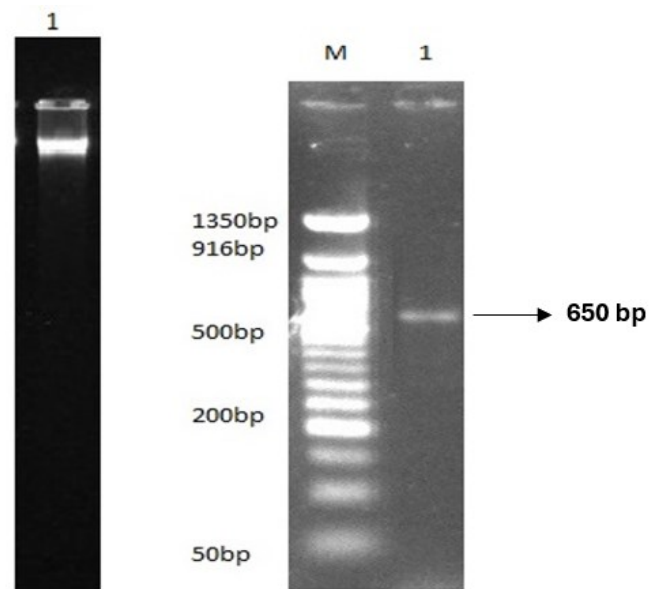


Figure 6: Agarose gel electrophoretogram. M is HyperLadder 1 kb Bioline, OS is the amplicon

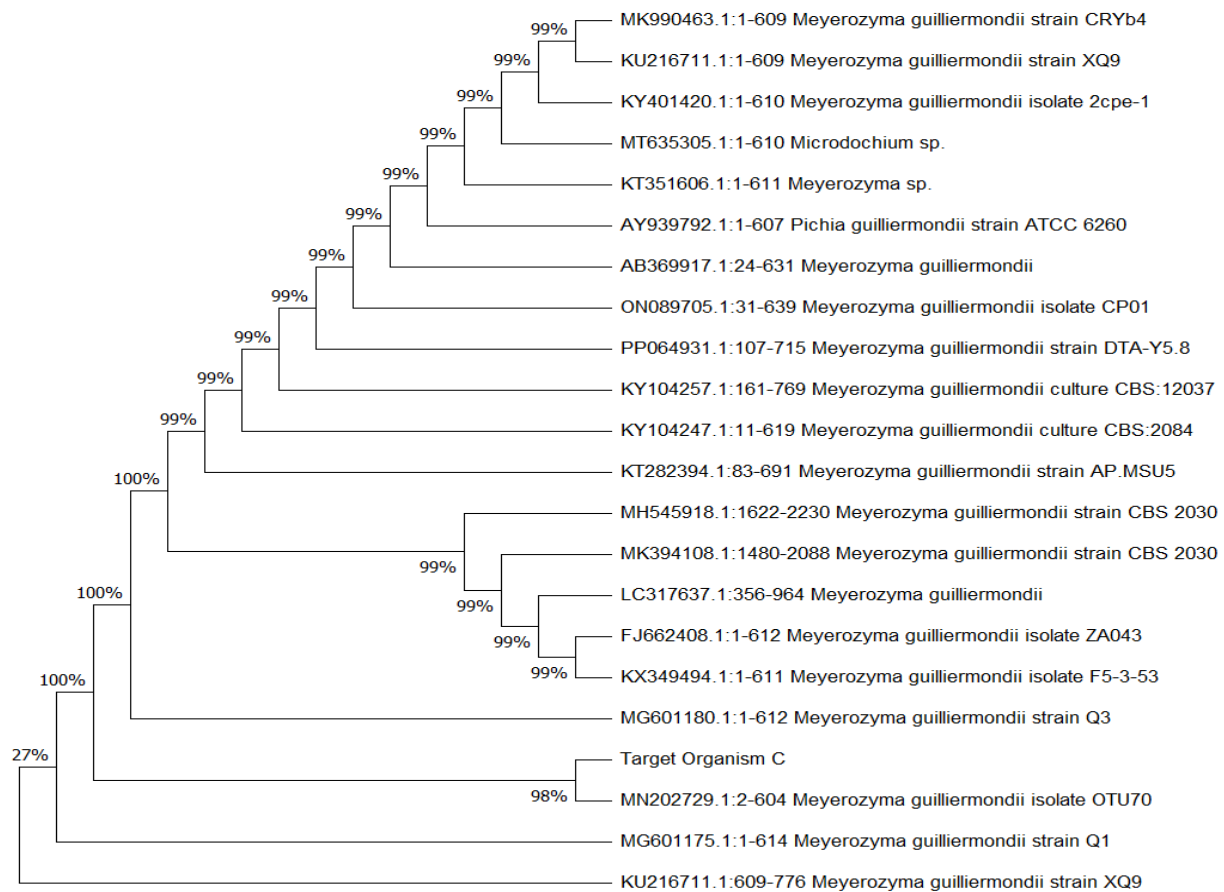


Figure 7: Phylogenetic tree of the fungal isolate

Table 2: Pure culture of isolated organisms



Pichia strain Y



Pichia strain Z



M. guilliermondii strain Q2

Plate 1: Pichia and M guilliermondii culture. Percentage sugar utilization for bioethanol production from sorghum by Pichia strain Y, Pichia strain Z and Meyerozyma guilliermondii strain Q2.

The biochemical process by which microbes break down sugars found in a substrate—such as sorghum—to create ethanol and other fermentation-related byproducts, such as organic acids, is known as sugar utilization. In this metabolic

process, complex sugars (like cellulose and hemicellulose in corn biomass) are broken down by enzymes into simpler sugars (like glucose and xylose), which are then taken up and used by yeast strains (like *Meyerozyma guilliermondii* and

Pichia) for growth and energy. The output and quality of the finished product are directly impacted by the efficiency of sugar utilization, which in turn affects the economic viability of producing bioethanol from agricultural feedstocks (Coulibaly et al., 2023). From the study, in consolidated bioprocessing (CBP) method, *Meyerozyma guilliermondii* strain Q2 (*M. guill.*) exhibited maximum percentage sugar utilisation of 71.283% on day 2, *Pichia* strain Z, had 53.738% on day 7 and *Pichia* strain Y had 48.382% on day 5 (Figure 8a). During simultaneous saccharification and fermentation (SSF) processes, *Meyerozyma guilliermondii* strain Q2 (*M. guill.*) and *Pichia* strain Y exhibited maximum percentage sugar utilisation of 70.088% and 52.325% on day 4 respectively, while *Pichia* strain Z utilised 53.688% on day 7 (Figure 8b). The result implies that *Meyerozyma guilliermondii* strain Q2 demonstrated higher ability to bio-convert released sugars from sorghum flour (starch) hydrolysis when compared to the two (2) *Pichia* strains. Rabah et al., (2020), reported 3.21% and 4.10% glucose yield

from Millet and Guinea corn husks respectively using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. For yeast strains to develop, be metabolically active, and be productive, they must use sugars efficiently. Sugars are utilized by microorganisms such as *Meyerozyma guilliermondii* and *Pichia* to produce ethanol, a valuable biofuel, and energy for cellular processes. While inadequate consumption may suggest metabolic inefficiencies or substrate constraints, yeast's ability to consume sugars effectively improves fermentation efficiency, leading to greater yields of desired products (e.g., ethanol in biofuel production) and improved overall process economics. Efficient use of sugar in biofuel production is essential for agricultural grains like corn, millet, and sorghum because it maximizes biomass conversion into bioethanol, improving the sustainability and economic viability of biofuel production systems and lowering the environmental impact of biofuel production.

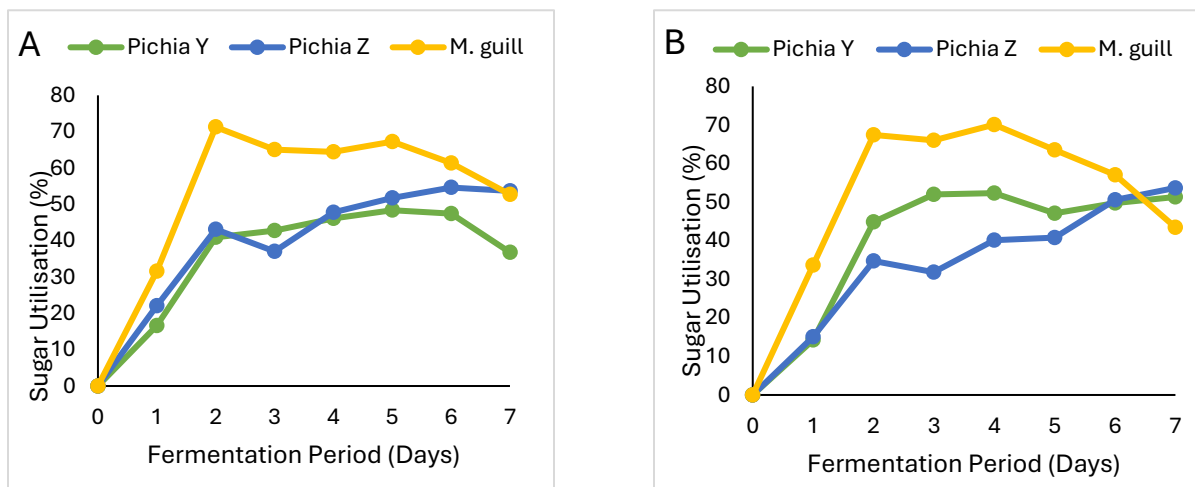


Figure 8: Percentage sugar utilisation in CBP (A) and SSF (B) of Sorghum flour

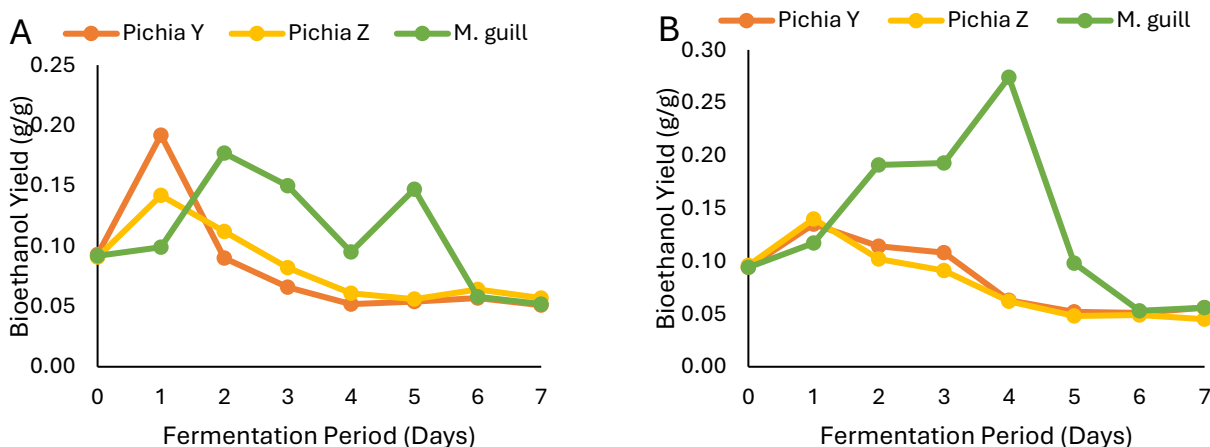


Figure 9: Bioethanol Yield (g/g) in (A) CBP and (B) SSF of Sorghum Flour

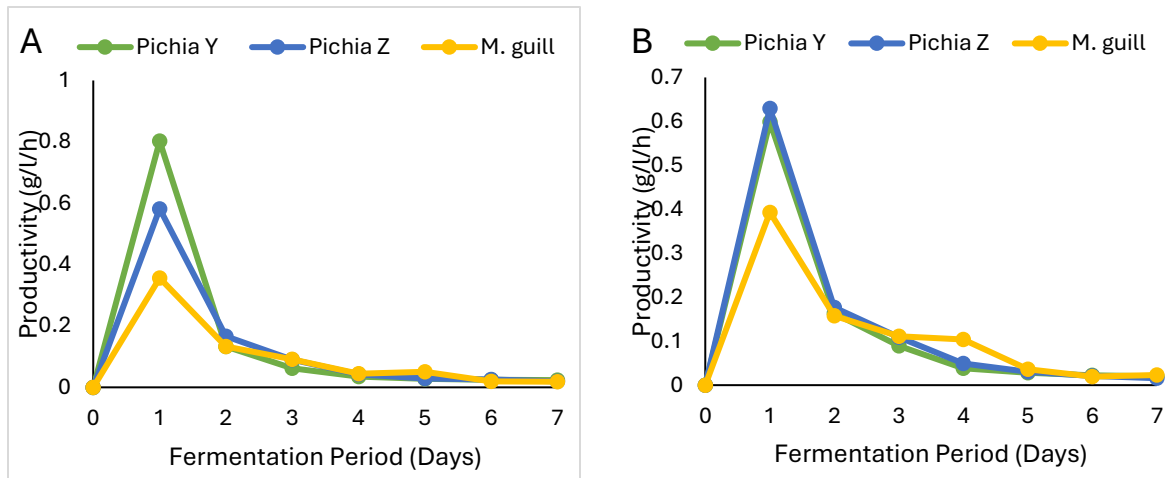


Figure 10: Bioethanol Productivity (g/l/h) in (A) CBP and (B) SSF of Sorghum Flour

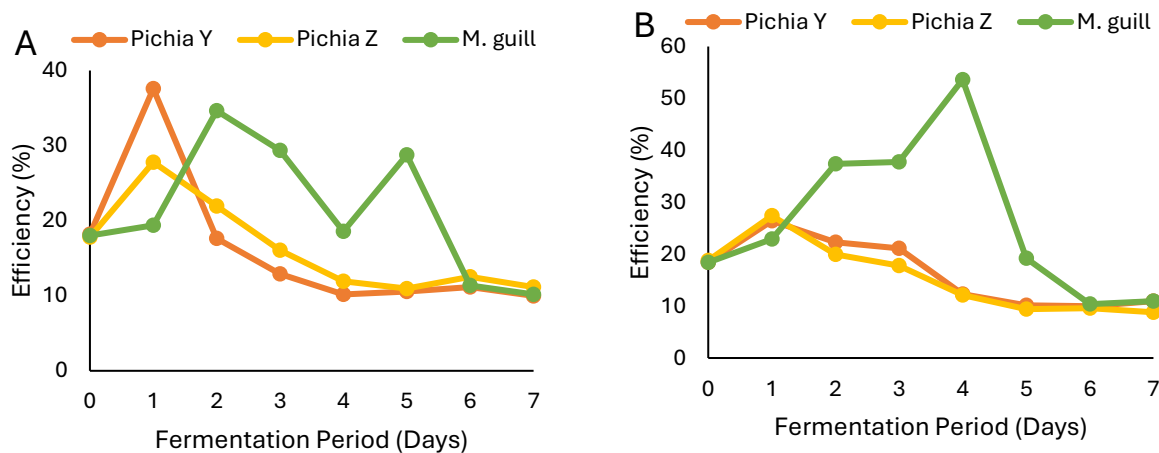


Figure 11: Fermentation Efficiency (%) in (A) CBP and (B) SSF of Sorghum Flour

Bioethanol yield

The quantity of ethanol generated per unit of substrate consumed, or grams of ethanol per gram of substrate, or g/g, is the standard definition of bioethanol yield, a critical measure in the assessment of bioethanol production systems. It functions as an important measure of the effectiveness and efficiency of the fermentation process, demonstrating the degree to which the microbes can transform the substrate's available sugars into ethanol (Liu et al., 2019). From the study, *Meyerozyma guilliermondii* strain Q2 produced the highest bioethanol yield of 0.274g/g on day 4, while *Pichia* strain Z and *Pichia* strain Y produced 0.140g/g and 0.135g/g on day 1 respectively, during simultaneous saccharification and fermentation (SSF). In CBP, *Pichia* strain Y produced the highest bioethanol yield of 0.192g/g on day 1, while *Meyerozyma guilliermondii* strain Q2 and *Pichia* strain Z produced 0.177g/g, and 0.142g/g on day 2, respectively (Figure 9). The above observation implies that, *Meyerozyma guilliermondii* strain Q2 demonstrated robust fermentative capabilities, when compared to *Pichia* strains Y and Z, during

SSF, likely due to its effective enzymatic systems for breaking down the complex carbohydrates in these grains and fermenting the resulting sugars into ethanol and the differences in the activities of the released enzymes from both organisms. The performance differences between the strains in SSF and CBP processes with sorghum flour suggests that *Pichia* strains Y and Z may be better suited to the conditions present in CBP, potentially due to differences in stress responses or enzyme production. Similarly, Hawaz et al. (2022), reported an ethanol yield of 0.29g/g using *Meyerozyma caribbica* MUTPm4 on bio-wastes and co-products of sugar factories after 48 hours of fermentation. Zheng et al., (2019) also reported bioethanol yield of 0.46g/g after 12 hours of fermentation using *Candida molischiana* and glucose as carbon source. This difference may be attributed to the type of yeast used and the direct availability of glucose for the fermentation process.

Bioethanol productivity

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Volumetric bioethanol productivity is calculated based on ethanol concentration produced per hour (g/L/h). It is dependent on the concentration of the carbon sources, and it measures how fast products are being produced. Higher bioethanol productivity values signify faster ethanol production rates, which are critical for optimizing industrial-scale bioethanol production processes. From the study, *Pichia* strain Y had maximum bioethanol productivity of 0.802g/l/h in CBP and 0.599g/l/h in SSF, *Pichia* strain Z gave 0.629g/l/h in SSF and 0.582g/l/h in CBP, and *Meyerozyma guilliermondii* strain Q2 gave 0.393g/l/h in SSF and 0.356g/l/h in CBP after 1 day of fermentation (Figure 10). *Pichia* strains Y and Z exhibited rapid ethanol production rates indicating robust enzymatic activities, efficient fermentation capacities, and ethanol tolerance, and *Meyerozyma guilliermondii* strain Q2, while exhibiting lower productivity rates compared to *Pichia* strains, contributed to ethanol production at a slower rate. This suggests differences in strain-specific metabolic pathways and fermentation efficiencies. Similarly, Malherbe et al. (2023), reported 0.68g/l/h and 0.42g/l/h in CBP and 0.35g/l/h and 0.40g/l/h in SSF of Sorghum flour after 96 and 48 hours of fermentation respectively, using two industrial *Saccharomyces cerevisiae* strains. Ochaikul et al., (2019) also reported 0.121g/l/h productivity after 24 hours of fermentation using *Candida shehatae* on base treated water hyacinth. These differences may be because of the pretreatment method and different substrate used, or the yeast strains used were unable to release sufficient reducing sugars for fermentation without the aid of external enzymes.

Fermentation efficiency

The ethanol yield is directly proportional to fermentation efficiency, the higher of ethanol yield, the greater fermentation efficiency will be achieved (Raharja et al., 2019). During alcoholic fermentation, the fermentable sugars are converted into ethanol and carbon dioxide by the action of microorganisms (Kechkar et al., 2019). Fermentation Efficiency is a critical metric in bioethanol production, quantifying the percentage of theoretical maximum ethanol yield achieved during fermentation and reflecting how effectively fermentable sugars in substrates are converted into ethanol by microbial strains within a specified timeframe. *Meyerozyma guilliermondii* strain Q2 exhibited a fermentation efficiency of 53.62% on day 4, while *Pichia* strain Z and *Pichia* strain Y exhibited 27.40% and 26.42% on day 1, respectively during simultaneous saccharification and fermentation (SSF). In CBP *Pichia* strain Y exhibited fermentation efficiency of 37.57% on day 1, while *Meyerozyma guilliermondii* strain Q2 and *Pichia* strain Z exhibited 34.64% and 27.79% on day 2 respectively (Figure 11). *Meyerozyma guilliermondii* strain Q2 consistently demonstrated higher efficiencies, indicating its potential for efficient bioethanol production from sorghum flour. In contrast, variations in efficiencies among *Pichia* strains reflect strain-specific differences in fermentation kinetics and

substrate utilization capabilities. Similarly, Malherbe et al. (2023) reported fermentation efficiencies of 79% and 24% in CBP and 41% and 23% in SSF of Sorghum flour after 96 and 48 hours of fermentation respectively, using two industrial *Saccharomyces cerevisiae* strains. Hashem et al. (2021) also reported fermentation efficiency of 77.5% after 72 hours of fermentation using *Pichia barkeri* on starchy biowaste.

Conclusion

Sorghum flour in this study has shown its potential application in Consolidated Bioprocessing and Simultaneous Saccharification and Fermentation process, using *Pichia* and *Meyerozyma* strains for bioethanol production. *Meyerozyma guilliermondii* strain Q2 has shown tolerance to sorghum flour and ethanol exhibiting maximum bioethanol yields after 96 hours of fermentation. The performance differences between the strains in SSF and CBP processes with sorghum flour suggests that *Pichia* strains may be better suited to the conditions present in CBP, potentially due to differences in stress responses or enzyme production. Optimisation and improved fermentation conditions for these yeasts can be of great importance for the increased production of ethanol using sorghum flour as substrate.

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