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Fungal biocatalytic valorization of sorghum: An integrated bottom-up and top-down framework for sustainable fermentable sugar production

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A B S T R A C T

This study presents an innovative research and optimization framework for maximizing fermentable sugar production from sorghum without any commercial media and chemical pre-treatment. It utilizes bottom-up and top-down approaches to establish a cost-effective and environmentally sustainable sorghum-based biorefinery. Leveraging the biocatalytic potential of *Aspergillus awamori* and *Aspergillus oryzae*, solid-state fermentation (SSF) and submerged fermentation (SmF) processes enhanced sugar yield. SSF emerged as the superior fermentation strategy, yielding an unprecedented total reducing sugar (TRS) concentration of 304.83 g/L (609.74 mg/g, db) from sorghum grain (particle size 1.18–0.6 mm) using *A. awamori* at 60 °C after 72 h of saccharification, achieving a saccharification efficiency of 83.17%. Optimized particle sizes of grain (1.18–0.6 mm) and bran (<0.6–0.3 mm) facilitated maximum TRS (304.83 g/L), α-amylase (982.5 ± 7.45 U/g), glucoamylase (10.93 ± 0.58 U/mL), and protease (168.05 ± 4.35 U/g, dry basis) production using *A. awamori* under solid-state fermentation. Notably, increasing saccharification temperature from 30 °C to 60 °C enhanced TRS yield 5.4-fold using SSF. Saccharification using fungal mash outperformed commercial enzyme cocktails, producing 1.2-fold higher TRS, demonstrating a low-cost alternative. Additionally, sorghum bran, a by-product constituting approximately 25% of the seed, exhibited substantial protease production (847.09 U/g, dry basis) by *A. oryzae* using bran particle sizes <0.6-0.3 mm, representing an 8.6-fold increase compared to *A. awamori* under solid-state fermentation (SSF). These findings demonstrate the transformative potential of fungal biotechnology in valorizing lignocellulosic biomass, fostering circular bioeconomy strategies, and renewable energy solutions. The proposed framework provides a scalable, green bioprocess for future industrial applications, contributing to sustainable agriculture and global energy security.

1. Introduction

The ongoing expansion of the global economy has led to a rise in energy consumption and community concerns regarding the accumulation of greenhouse gases in the atmosphere and their impact on climate change. Among renewable energy sources, biofuels have emerged as a promising alternative due to their non-toxic, compostable, and carbon-neutral attributes [1–4]. Wheat, corn, rice, and barley are widely utilized as feedstocks for bioethanol production, while sorghum remains relatively underexplored, despite having a comparable starch content (68–74 %) to wheat and corn [5,6]. Sorghum offers several advantages over other cereals, including its adaptability to various environmental stresses, such as high temperatures, water scarcity, and low fertilizer costs, making it a suitable crop for cultivation in marginal lands [6–8]. Furthermore, sorghum is the second cereal crop to undergo genome sequencing, providing diverse genetic opportunities for breeding and renewable applications [9]. Despite its promising attributes, sorghum remains underutilized for sugar and biofuel production compared to wheat, corn, and rice. According to data from ScienceDirect, only 198 research articles on sorghum, sugar, and biofuel were published in 2024, compared to 753 and 624 articles on sugar and biofuel production from corn and wheat, respectively. This disparity highlights the need for a comprehensive exploration of sorghum's potential, particularly in biofuel production, to unlock its capabilities compared to more extensively studied crops.

Aspergillus fungal strains play a crucial role in starch and biomass hydrolysis due to their ability to secrete a diverse range of hydrolytic enzymes, including amylases, cellulases, and proteases, which efficiently break down complex polysaccharides into fermentable sugars, similar to commercial enzyme treatments [10]. Fungi are well adapted

Abbreviations: SSF, Solid state fermentation; SmF, Submerged fermentation; TRS, Total reducing sugars; FAN, Free amino nitrogen; FTIR, Fourier transform infrared spectroscopy; PSD, Particle size distribution; SF, Sorghum flour; WF, Wheat flour; RMP, Revolutions per minute; TS, Total starch; CEC, Commercial enzyme cocktail.

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to efficiently degrade biomass, as it constitutes fungi's primary source in their natural environments [11]. Fungal-based hydrolysis presents an efficient, low-cost, and environmentally friendly alternative to conventional enzymatic hydrolysis. Extraction of fermentable sugars from sorghum grain via fungal hydrolysis for biofuel production also generated nutrient-rich non-fermented solids with high protein and free amino nitrogen (FAN) as a byproduct, enhancing their commercial value beyond conventional dried distillers' grains (DDG) [12]. Fungal-based saccharification and biotransformation without using any commercial chemicals not only improved the overall economic feasibility of the process but also contributed to a zero-waste biorefinery model (top-down approach) by repurposing solid residues as high-protein animal feed.

Previous studies have explored wheat and corn-based biorefineries, with research focusing on fungal submerged fermentation (SmF) for nutrient-complete fermentation substrates [13,14] and integrated wheat-based biorefineries from flour to value-added chemical production [15]. Building on these foundations, Du, et al. [16] applied solid state fermentation (SSF) using *A. awamori* and *A. oryzae* on wheat bran to generate enzyme-rich hydrolysates for starch and protein breakdown. Barcelos, et al. [17] reported a maximum total reducing sugar (TRS) yield of 193.4 g/L from sorghum grain (0.3 mm) using commercial enzymes without fungal treatment, but their study lacked comparisons between solid-state and submerged fermentation. Similarly, Ren, et al. [18] and El-Imam, et al. [19] investigated TRS production from sorghum grain (0.2–0.25 mm) and sorghum husk (<1 mm), respectively, without assessing the impact of different particle sizes or fermentation methods on TRS yield. However, no studies have systematically investigated the effect of particle size distribution (PSD) on sorghum grain and bran for TRS production using SSF and SmF, nor have they explored critical process parameters such as pH deviation, water loss after autoclaving the media, and *Aspergillus* growth patterns on different particle sizes and substrates in sorghum-based biorefineries.

To address these research gaps, this study develops a novel comprehensive approach to identify the optimal combination of substrate, particle size, fermentation mode, and fungal strain for maximizing TRS production from sorghum without the use of chemical treatment or commercially available enzymes. This process follows a bottom-up approach to establish a cost-effective and environmentally sustainable sorghum-based biorefinery. The bottom-up biorefinery approach involves transforming existing biomass processing facilities, originally designed to produce one or a few products, into more integrated systems by incorporating additional technologies to expand the product range and enhance biomass utilization [20,21]. In this context, sugar production for biofuel from sorghum grain exemplifies a bottom-up strategy, where traditional grain processing is extended through enzymatic hydrolysis and fermentation to yield fermentable sugars for bioethanol and other bio-based products. Additionally, utilizing sorghum bran and grain to produce free amino nitrogen (FAN), protease, and α-amylase exemplifies a top-down biorefinery approach, where all biomass fractions are fully exploited to generate multiple high-value products, thereby minimizing waste. This strategy aligns with the zero-waste objective of top-down biorefineries. Notably, the fungal treatment generates a solid waste fraction rich in protein and short-chain fatty acids [22], which can be repurposed as feed for cattle and fish, aligning with a zero-waste biorefinery (top-down) model [20,23]. The specific objectives of this study include (1) comparing TRS yield using SSF and SmF, (2) evaluating the effects of particle size and substrate, (3) assessing the saccharification efficiency using fungal and commercial enzyme cocktails, (4) parameters screening and correlation scoring with TRS yield, and (5) process optimization and validation.

2. Materials and methods

The overall experimental design is illustrated in Fig. 2.

2.1. Raw materials

Sorghum grain and bran were used as the primary substrates for fungal fermentation and for fermentable sugar production (raw material for biofuel production), while commercial whole-meal sorghum and wheat flour served as controls. Raw sorghum (*Sorghum bicolor*) grain (red color) was collected from the local region of Toowoomba, Queensland, whereas wheat flour was purchased from a local supplier (Powlett Hill, Victoria, Australia), and sorghum flour from "The Source Bulk Foods" in Australia.

2.2. Grain milling and particle size distribution (PSD)

Sorghum grain was heated at 80 °C for 1 h in a drying oven prior to grinding, and no visible changes in color or structural morphology were observed during heat treatment. The treated grain was then milled using a kitchen blender, followed by size fractionation through mesh sizes of 1.18 mm, 0.6 mm, and 0.3 mm using a mechanical sieve shaker (Endecotts, EFL 2000) to obtain different particle size distributions (PSD). (see supplementary Fig. S1). The grain was classified into four (04) size categories: >1.18 mm, 1.18–0.6 mm, <0.6–0.3 mm, and < 0.3 mm, while the bran was separated into three (03) size fractions: 1.18–0.6 mm, <0.6–0.3 mm, and < 0.3 mm. The surface morphology of the grain particles was analyzed using Scanning Electron Microscopy (SEM) (JEOL JCM-6000, Japan) (Fig. 1). These particle size fractions were systematically investigated to assess their impact on the studied parameters (SSF vs. SmF, fungal strains) for TRS production. From 100 g of raw sorghum seeds, 75.39 g of grain and 24.61 g of bran were recovered, indicating the proportional distribution of these fractions.

2.3. Initial characterization of sorghum grain and bran

Bulk density was determined for solid substrates, including wheat flour, sorghum flour, different particle size fractions of sorghum grain, and bran. The bulk density (ρ_b) of solid particles was calculated according to the method described by Abalone, et al. [24] using the Eq. (1). Particle density (ρ_p) and porosity (ε) were measured using the gas pycnometer (AccuPyc III, model: 3150), as outlined in ASTM [25,26], and calculated using Eqs. (2) and (3), respectively. Bed tortuosity (τ) (without dimension) has been calculated using Eq. (4) [27]. The moisture content (MC) of different size fractions of sorghum grain and bran, prior to fungal fermentation, was observed to range from 4.72 % to 8.16 % (dry basis) and from 4.74 % to 8.51 % (dry basis), respectively, using a moisture analyzer (Ohaus MB120). Details are mentioned in Table 1.

$$\text{Bulk density}(\rho_b) = \frac{mass\ of\ dry\ solid\ substrate\ (g)}{total\ volume\ of\ solid\ substrate\ and\ air(mL)} \quad (1)$$

$$\text{Particle density}\ (\rho_p) = \frac{weight\ of\ grain\ fraction\ (g)}{true\ volume\ of\ grain\ fraction\ (mL)} \quad (2)$$

$$\text{Porosity}\ (\varepsilon) = 1 - \left(\frac{Bulk\ density}{particle\ density}\right) x\ 100 \quad (3)$$

$$\text{Tortuosity}\ (\tau)^2 = \varepsilon^{-1/2} \quad (4)$$

Total starch estimation was performed as per the protocol of the Starch Assay Kit (MAK368, Sigma-Aldrich). The starch concentration was determined based on a standard curve: y = 3.1112x and R^2: 0.9988 (See supplementary Fig. S2).

2.4. Microorganisms

Aspergillus awamori and *Aspergillus oryzae* were utilized for the production of an enzyme cocktail comprising α-amylase, glucoamylase, and protease, which facilitates the saccharification of sorghum grain and bran. *A. awamori* (2342™, NRRL 3112) was obtained from the CSIRO

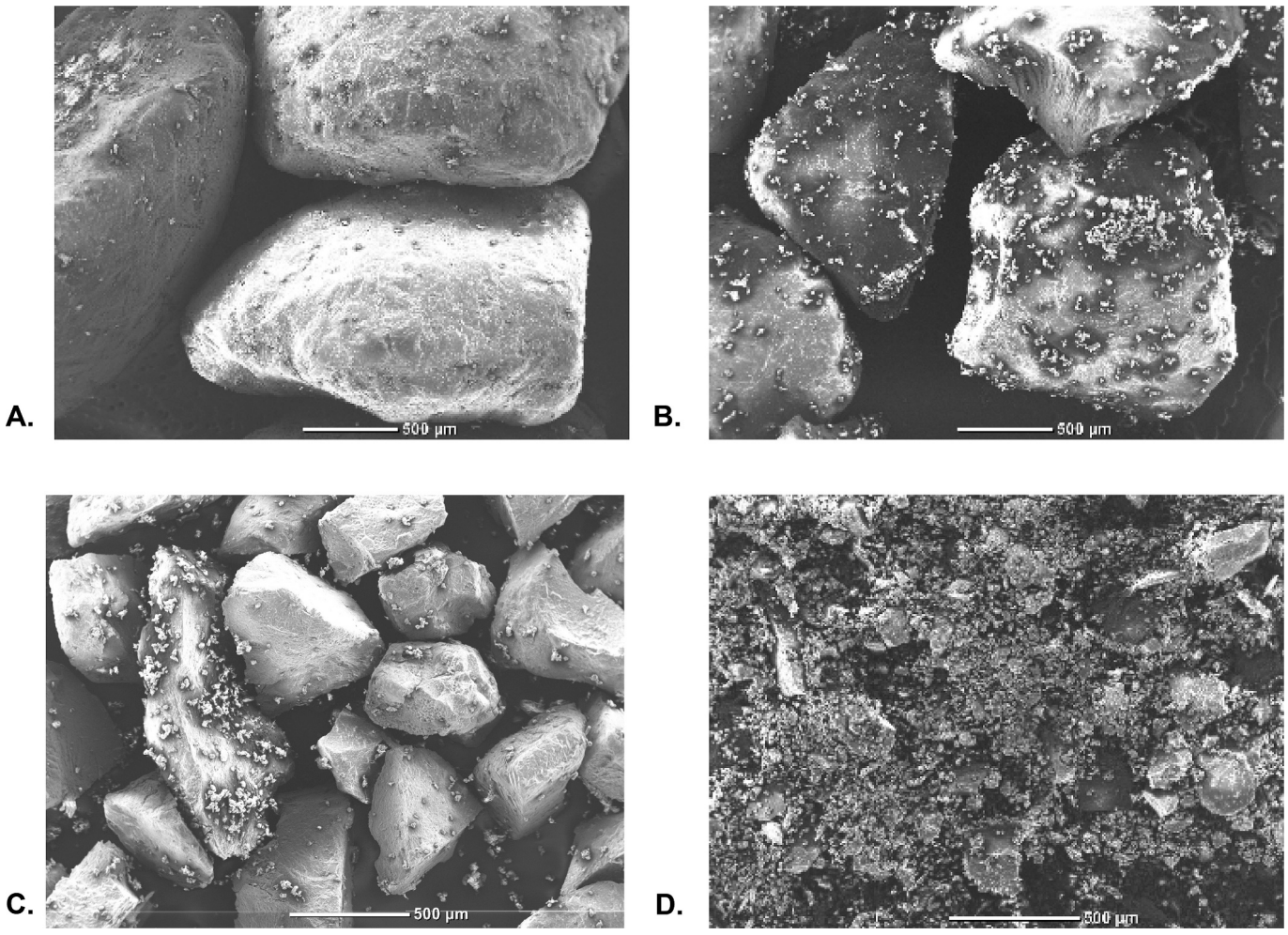

Fig. 1. Scanning electron microscopy (SEM) of sorghum grain (40× magnification) (A) PSD >1.18 mm (smooth surfaces with high void spaces). (B) 1.18–0.6 mm (fractured surface with attached fine particulate matter). (C) <0.6–0.3 mm (fractured surface and irregular shapes). (D) <0.3 mm (highly compact with minimal void spaces).

culture collection, while *A. oryzae* was sourced from Koji starter (rice culture, Australia). Both strains were initially stored as dry spores in sand at 4 °C. Before experimental use, *A. awamori* and *A. oryzae* spores were purified and prepared according to the method described by Koutinas, et al. [28] and Abdul Manan and Webb [29], with slight modifications. Pure cultures obtained from the supplier were streaked onto 90 mm Petri dishes containing 4 % (w/v) agar and incubated at 30 °C for 7 days to allow for sporulation. Single spores were then isolated under sterile conditions, transferred to fresh agar plates, and examined using a light microscope to confirm morphological characteristics and the absence of contamination. Sporulation was monitored during the incubation period, and well-sporulated cultures were selected. The resulting pure spore cultures were maintained on agar slopes at 4 °C for future use.

2.4.1. Inoculum preparation of A. awamori and A. oryzae

After autoclaving, 4 % Potato Dextrose Agar (PDA, Sigma Aldrich) was distributed into 90 mm Petri dishes inside a biosafety cabinet (Esco, class II BSC) and allowed to solidify. Fungal spores from stock cultures were streaked onto the plates under aseptic conditions, and the plates were covered, labeled, and incubated at 30 °C for up to seven days [29]. For the preparation of a working fungal spore bank, a small amount (0.5 mL) of the spore suspension was spread onto the surface of a 50 mL solid medium (8 % sorghum +2 % agar) in 500 mL Erlenmeyer flasks (total number of flasks was 10), and the inoculated flasks were incubated at 30 °C for five (05) days. After incubation, 100 mL of 1× PBS buffer and glass beads were added to the first flask, and the spores were suspended by gentle shaking [28]. The same suspension was sequentially used to wash up to the tenth flask, resulting in a highly concentrated spore suspension (see supplementary Fig. S3).

2.4.2. Microscopic observation and spore count

The morphology of spores was observed using a light microscope (Nikon Eclipse E600) with a magnification of 100× (supplementary Fig. S4). This not only observed the morphology but was also used to check for bacterial contamination. Spore counting was performed using a Neubauer hemocytometer, yielding a concentration of 2.37×10^8 spores/mL for *Aspergillus awamori* and 1.01×10^8 spores/mL for *Aspergillus oryzae*.

2.5. Solid state fermentation (SSF) using A. awamori and A. oryzae

For SSF and trend analysis of *A. awamori* growth over nine (09) days, nine (09) Petri dishes were prepared for each sample, including seven (07) different size fractions of sorghum (grain and bran) and two (02) controls (commercial sorghum flour and commercial wheat flour), resulting in a total of nine (09) samples (Table 1). Thus, 81 plates were prepared per replicate, with each plate containing 10 g (dry weight) of the substrate (sorghum grain or bran) and adjusted to 75 % moisture content (Mc) before autoclaving, following the Eq. (5) [30].

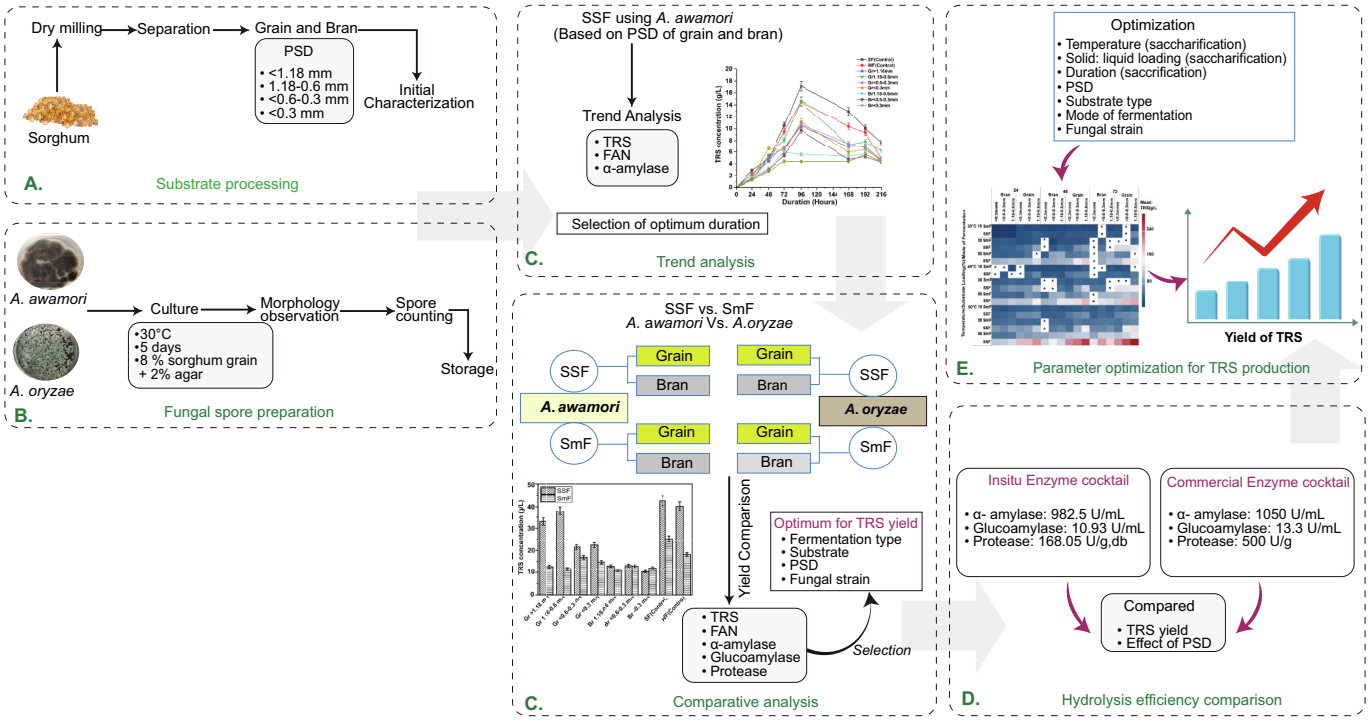

Fig. 2. Schematic representation of the experimental workflow in this study. [PSD: particle size distribution; TRS: total reducing sugars; FAN: free amino nitrogen].

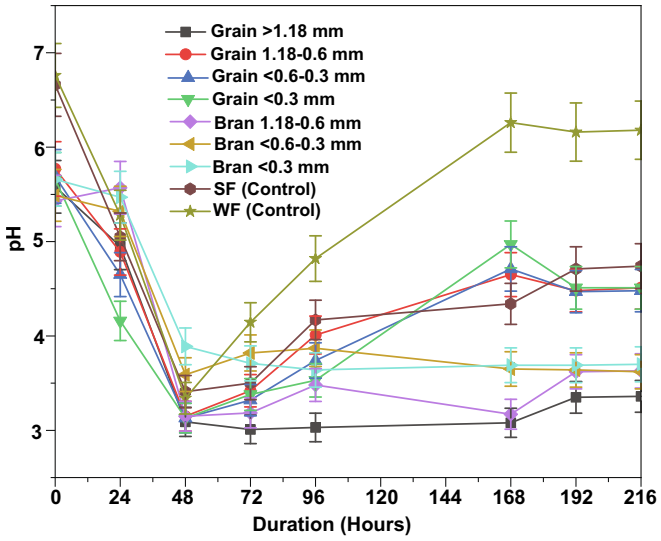

Fig. 3. pH fluctuation during SSF with *A. awamori* [SF: Commercial sorghum flour; WF: commercial wheat flour].

$$\text{Mc}\ (\%) = \frac{W}{W+C}\ \text{X}\ 100 \qquad (5)$$

Where, W: Weight of water (g); C: Weight of dry sorghum grain/bran (g).

Three (03) replicates were prepared for *A. awamori*, resulting in a total of 243 plates. After autoclaving at 121 °C for 15 min, each plate was inoculated with *A. awamori* (10^8 spores/10 g solid substrate) from a previously prepared working spore bank inside the biosafety cabinet. The plates were incubated at 30 °C and three plates from each sample were sacrificed daily to determine total reducing sugars (TRS), FAN, and α-amylase activity, assessing the optimum duration for maximum yield (TRS and enzymes), particle size, and substrate type (bran or grain) by using trend analysis (Fig. 4). Based on the optimum duration of *A. awamori*, three (03) replicates were set up for *A. oryzae* (27 plates in total), and inoculated with 10^8 spores/10 g of solid substrate same as *A. awamori*. TRS and enzyme activity were estimated after five (05) days of fermentation and subsequently compared with the performance of *Aspergillus awamori* to evaluate relative efficacy.

2.6. Submerged fermentation (SmF) using A. awamori and A. oryzae

Submerged fermentation (SmF) was conducted in a 250 mL Erlenmeyer flask in triplicates. Each 250 mL flask contained 10 % (w/v) substrate (sorghum flour/bran) without the addition of any other nutrients or chemicals, similar to the SSF method. The fermentation media were sterilized at 121 °C for 15 min and cooled before inoculation with *A. awamori* and *A. oryzae* at a ratio of 1×10^7 spores per gram of dry-weight sorghum substrate [31]. Fermentation was carried out in an orbital shaking incubator (Ratek OM25) at 30 °C and 200 rpm for five days.

2.7. Saccharification of sorghum grain and bran with in-situ enzyme cocktail

A. awamori and *A. oryzae* were employed in both solid-state fermentation (SSF) and submerged fermentation (SmF) to produce amylolytic (α-amylase, glucoamylase) and proteolytic enzymes using sorghum as a substrate. The in situ-produced enzyme cocktail was then utilized for the saccharification of starch from sorghum grain and the lignocellulosic fraction of sorghum bran to generate total reducing sugars (TRS). For SSF, fermented solid media from each Petri dish was mixed with distilled water at a ratio of 10 g of solids to 50 mL of distilled water (no minerals), and saccharification was conducted in a 250 mL Duran bottle (no addition of water) at 30 °C for 60 min, with shaking using an automated shaker at 180 rpm.

2.7.1. Extraction (Enzymes and total reducing sugars)

After saccharification, the mixture was centrifuged at 5000 ×*g* for 10

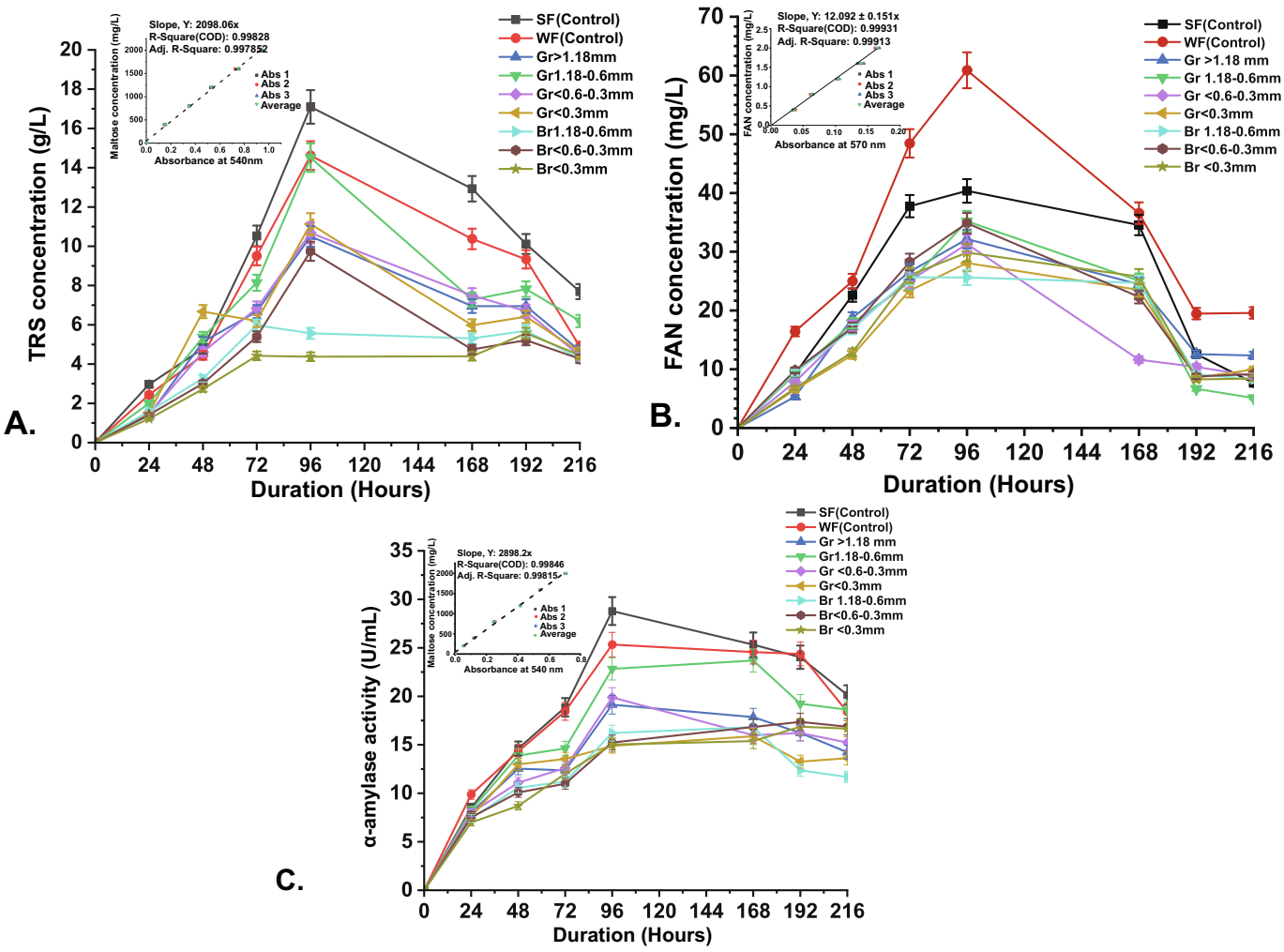

Fig. 4. Trend analysis of *Aspergillus awamori* in solid-state fermentation (SSF) for (A) TRS production, (B) FAN production, and (C) α-amylase production. [SF: commercial sorghum flour; WF: commercial wheat flour; Gr: grain and Br: bran] [Note: Solid-liquid ratio: 1:20].

min, and the supernatant was filtered through Whatman No. 1 filter paper to collect medium content with TRS and enzyme cocktail. For SmF, the fermentation broth was directly centrifuged at 5000 ×*g* for 10 min, and the supernatant was collected and filtered. Supernatants from both methods (SSF and SmF) were used for additional analysis and stored at 4 °C for future use. After comparing the enzyme (α-amylase, glucoamylase, and protease) activity and TRS production efficiency of the two fermentation methods (SSF vs. SmF), the study further optimized TRS production by systematically evaluating key parameters, including saccharification temperature (30 °C - 60 °C), saccharification duration (24–72 h), mode of fermentation (SSF Vs SmF), particle size distribution (7 different PSD), fungal strain (*A. awamori* Vs *A. oryzae*), and solid: liquid (1:2; 1:4; and 1:10, w/v) ratio.

2.8. Process optimization for TRS production

2.8.1. Saccharification with fungal mash and comparison with commercial enzymes

Fungal mash from SSF was used for the saccharification of sorghum grain instead of a liquid enzyme cocktail. TRS and FAN production from both grain and bran were analyzed after five (05) days of fungal culture, and the saccharification activity of the fungal-produced enzyme cocktail was compared with the commercial enzyme cocktail. Initially, 2.5 g of fungal mash was collected after five (05) days of *A. awamori* solid-state fermentation (SSF) using sorghum grain (particle size: 1.18–0.6 mm) as a source of enzyme cocktail. The collected mash was then subjected to saccharification for 24 h at 60 °C with continuous shaking at 180 rpm, and a 1:2 solid: liquid ratio was maintained, resulting in the production of 982.5 U/mL of α-amylase, 10.925 U/mL of glucoamylase, and 168.05 U/g (dry basis) of protease. Adefila, et al. [32] reported a maximum α-amylase activity of 1710 U/mL from sorghum grain under optimized conditions and Makanjuola, et al. [31] achieved a peak glucoamylase activity of 19.3 U/mL from sorghum bran after process optimization. In parallel, a commercial enzyme cocktail containing 1050 U/mL of α-amylase (10065-10G, Sigma Aldrich), 13 U/mL of glucoamylase (A7095-50 mL, Sigma Aldrich), and 500 U/g of protease (P6110-50 mL, Sigma Aldrich) was prepared (Table 3). Saccharification experiments were conducted in two separate flasks: one using 2.5 g of fungal mash per 5 g of raw substrate, considered as an in-situ enzyme cocktail, and the other with the commercial enzyme cocktail (all saccharification experiments and optimization results were calculated by subtracting the initial TRS value present in the 2.5 g mash, as mentioned in Table 3, to ensure accurate estimation of saccharification efficiency). Three (03) replicates were performed to ensure the accuracy. The experiments were carried out at 60 °C for four (04) days with a solid-to-liquid ratio of 1:2. To enhance enzymatic activity, tap water was used, and continuous

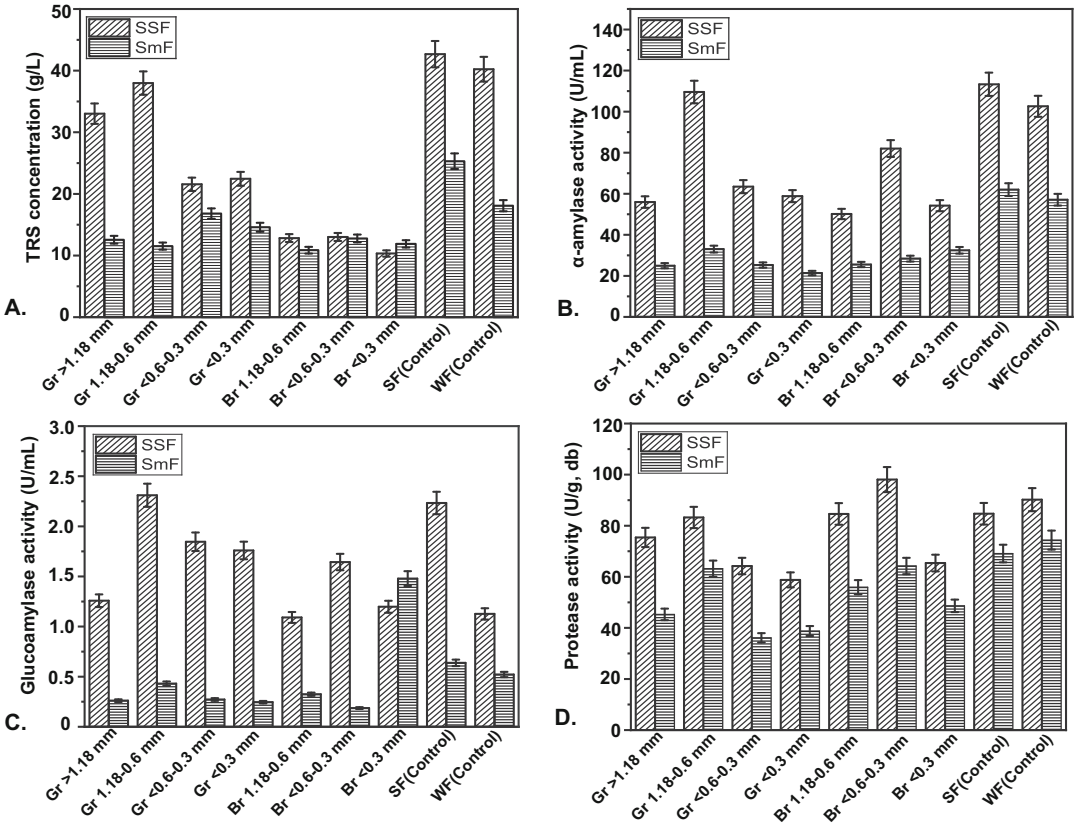

Fig. 5. SSF vs SmF yield analysis using *A. awamori*: (A) Total reducing sugars (TRS) production, (B) α-amylase activity, (C) Glucoamylase activity, and (D) Protease activity. [Note: solid-liquid ratio (SSF): 1:8 (w/v)].

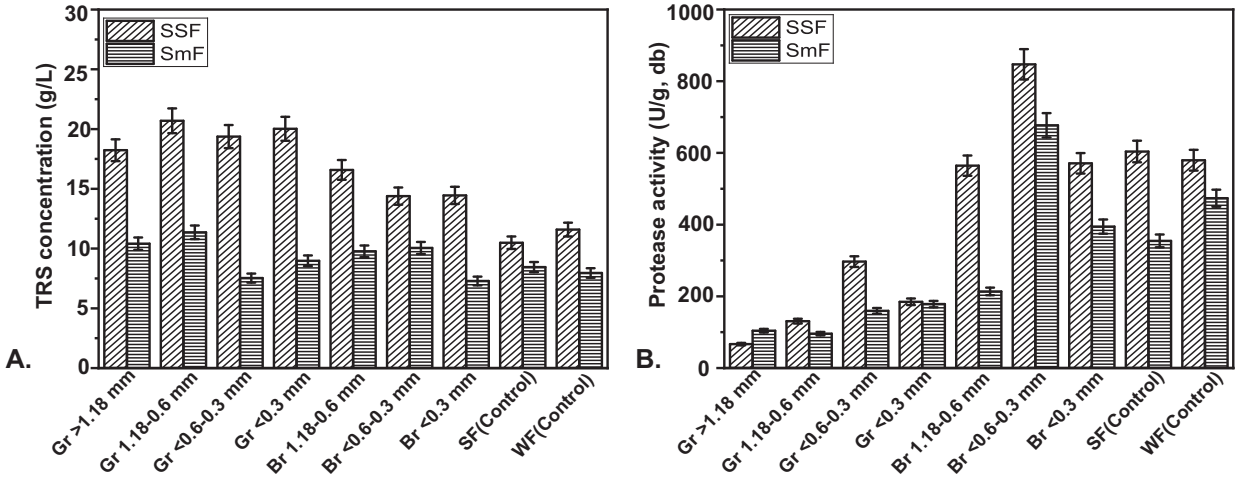

Fig. 6. SSF vs SmF yield analysis using *A. oryzae*: (A) Total reducing sugars (TRS) production, and (B) protease activity. [Note: solid-liquid ratio (SSF): 1:8 (w/v)].

mixing was maintained using shaking incubator (Ratek, OM25) set at 180 RPM. Saccharification efficiency for the in-situ enzyme complex was calculated using the following Eq. (6) [31]

$$\text{Saccharification efficiency } (\%) = \frac{\textit{Weight of TRS } (g)}{\textit{Weight of grain or bran } (g) \times \textit{starch content } (\%) X\ 1.11} \times 100 \quad (6)$$

2.8.2. Temperature and solid: liquid loading

Based on the findings of previous experiments detailed in Sections 2.5 and 2.6, the best fermentation method (SSF), substrate (sorghum grain), and particle size range (1.18–0.6 mm), fungal strain (*A. awamori*) were selected for further saccharification studies. These parameters

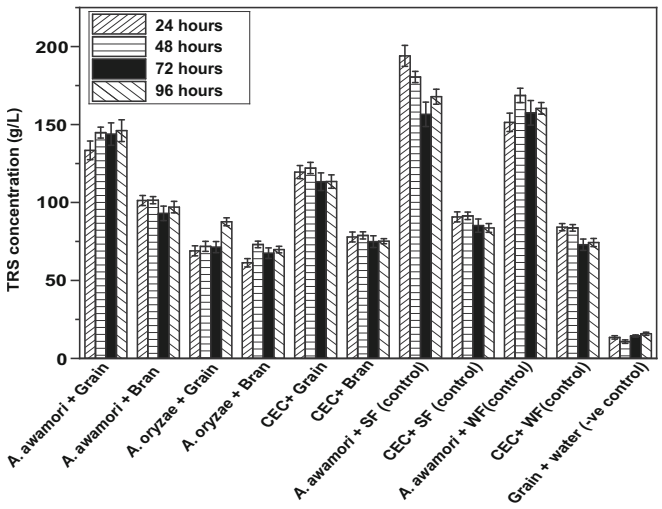

Fig. 7. Comparison of saccharification efficiency between in situ produced enzymes and commercial enzyme cocktails based on total reducing sugar (TRS) production at 60 °C. [Note: Grain: PSD 1.18–0.6 mm; Bran: PSD <0.6–0.3 mm; CEC: commercial enzyme cocktail; SF: Commercial sorghum flour used as a positive control; WF: commercial wheat flour used as a positive control, -ve: Negative].

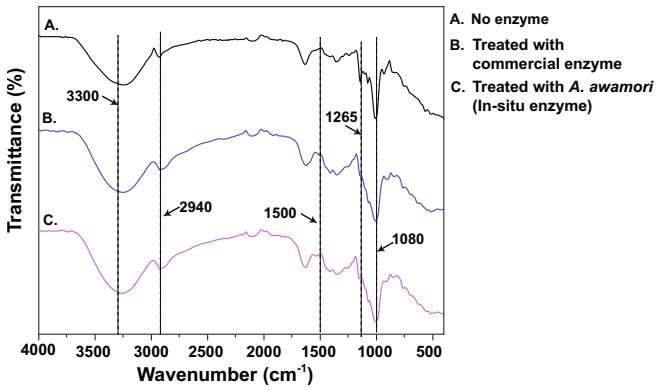

Fig. 8. FTIR spectra of sorghum grain [untreated grain (A. black line), grain treated with commercial enzymes (B. blue line), and grain treated with in situ enzyme produced by Aspergillus awamori (C. pink line). The x-axis represents wavenumber (cm^{-1}), and the y-axis represents transmittance (%)].

were fixed during the saccharification experiments to maintain consistency and focus the optimization on key processing conditions. To optimize saccharification for enhanced TRS production, three variables were systematically studied: temperature, solid: liquid ratio, and incubation duration. Specifically, four (04) different temperatures (30 °C, 40 °C, 50 °C, and 60 °C) and three (03) different solid: liquid loading ratios: 1:2 ratio (w/v), 1:4 ratio (w/v), and 1:10 ratio (w/v) were investigated to identify the most effective condition for enhance TRS production at maximum level. Tap water was used as the medium for the optimization process, with a saccharification duration of 4 days; the results from each day were analyzed.

2.9. Analytical methods

Total reducing sugar (TRS) released after saccharification was estimated using the DNS (3,5-dinitrosalicylic acid) method (slope of calibration curve: y: 2098.06x; R^2: 0.99828, coefficient of variance: 2.01 %) according to Miller [33]. The free amino nitrogen (FAN) concentration in liquid samples was determined using the ninhydrin colorimetric method with a coefficient of variance (COV) is equal to 3.92 %, as outlined in the European Brewery Convention [34] and Koutinas, et al. [15], with a standard curve equation, y: 12.09 ± 0.151x; R^2: 0.099931. α-amylase activity was estimated using the Miller [33] and Vidyalakshmi, et al. [35] method, where one unit of α-amylase was defined as the amount of enzyme required to liberate 1 mg of maltose from starch within 3 min at 20 °C. The maltose standard curve for α-amylase estimation was obtained with the equation y: 2898.2x; R^2: 0.99846, and α-amylase activity was calculated using the following Eq. (7).

$$\text{Units} \Big/ \text{mL} = \frac{mg\ of\ maltose\ released\ x\ Dilution\ factor(DF)}{mL\ of\ enzyme\ x\ reaction\ time} \quad (7)$$

Glucoamylase activity was assayed as described by Koutinas, et al. [28], where one unit (U) of glucoamylase was defined as the amount of enzyme required to generate 1 mg of glucose per minute under the assay conditions. Protease activity was determined following the method of Wang, et al. [36], where one unit (U) of protease was defined as the amount of enzyme required to liberate 1 mg of free amino nitrogen (FAN) per minute under the assay conditions. TRS, FAN, and enzyme activity (α-amylase, glucoamylase, and protease) were determined using a UV spectrophotometer (Hatch, DR 6000). Fourier-transform infrared spectroscopy (Shimadzu, GladiATR 10) was used for qualitative analysis of sorghum grain starch degradation patterns.

2.10. Data analysis

Statistical analysis was performed using OriginPro 2025 and IBM SPSS (version 29.0.1.0) application software. The data are presented as the mean of triplicate experiments for each treatment. Mean values and standard deviations were computed using Microsoft Excel (Version 2408). Correlation, heatmap, and contour plots were analyzed using Minitab Version 2022. The level of statistical significance was set at $p \leq 0.05$.

3. Results and discussion

3.1. Physicochemical characterization of sorghum

The particle size distribution (PSD) of sorghum grain and bran plays a crucial role in determining their physical properties and initial biochemical composition. One of the most significant physical factors is particle size, which directly influences the surface area-to-volume ratio of the solid substrate [37]. An optimal particle size is essential to balance mycelial growth and ensure adequate oxygen and nutrient availability [38]. Furthermore, particle size impacts the size of inter-particle voids and the overall porosity of the substrate, which are critical for mass transfer and microbial activity during fermentation processes [39]. While numerous studies have explored particle size distribution (PSD) in the context of wheat fermentation, there is a notable lack of comprehensive data on sorghum grain, particularly in sorghum base fermentation and biorefinery [6].

This study addresses this gap by focusing on the development and optimization of total reducing sugars (TRS) production, utilizing sorghum with varying particle sizes and comparing two distinct fermentation modes: solid-state fermentation (SSF) and submerged fermentation (SmF) using A. awamori and *A. oryzae*. This study investigated four (04) different particle sizes of sorghum grain (>1.18 mm, 1.18–0.6 mm, <0.6–0.3 mm, and <0.3 mm) and three (03) particle sizes of sorghum bran (1.18–0.6 mm, <0.6–0.3 mm, and <0.3 mm). Smaller particle sizes (<0.3 mm) of both grain and bran exhibited higher porosity (Ɛ) (66.85–74.4 %), lower bulk density (ρ_b) (0.366–0.486 g/cm^3), and lower tortuosity (τ) (0.341–0.349) compared to larger particles. Small particle size grain and bran showed higher initial moisture contents (8.16–8.51 %) compared to large particle size (4.71–6.11 %) (details in Table 1). The increased surface area-to-volume ratio of smaller particles enhances water retention by providing more contact

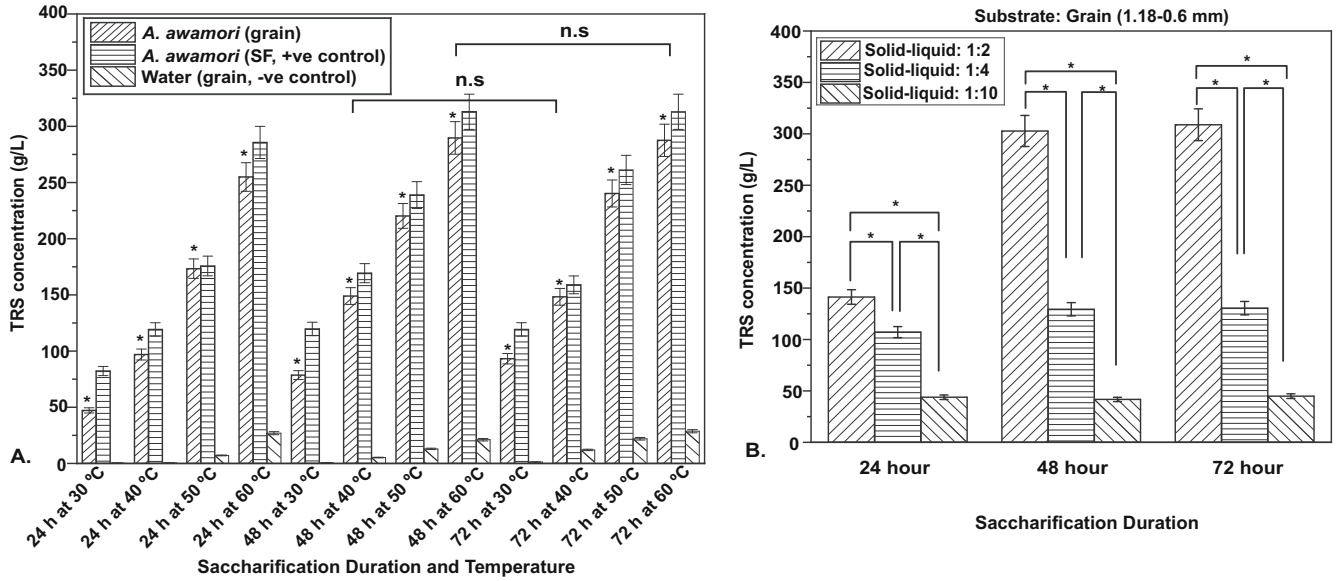

Fig. 9. Optimization of TRS yield using SSF: (A) effect of saccharification duration and temperature [*: statistically significant, where $p < 0.001$; n.s: not significant; +ve: positive; −ve: negative. Significance testing was performed within the sample group (grain) only]. (B) Effect of solid: liquid loading [*: statistically significant, $p < 0.001$].

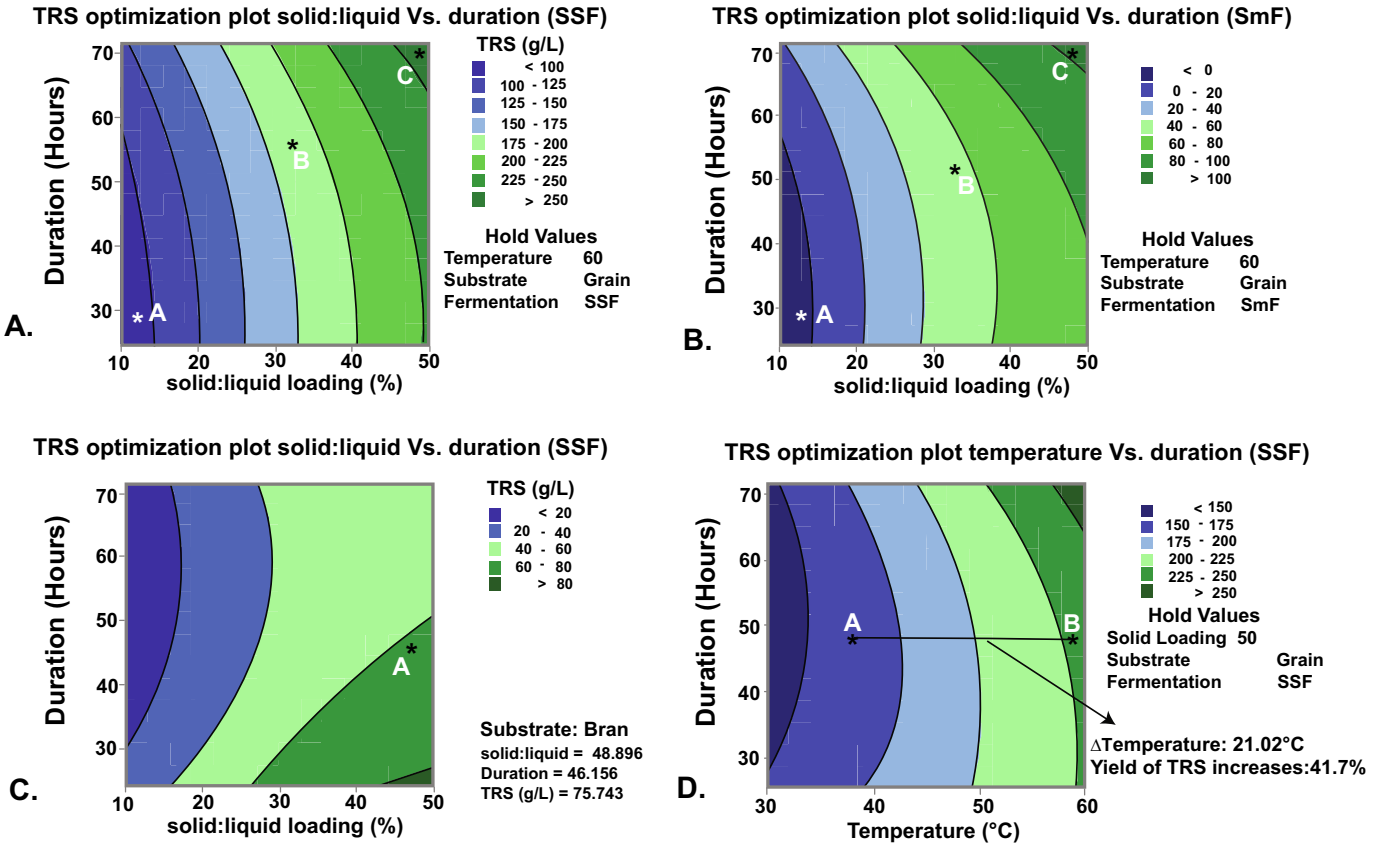

Fig. 10. Contour plots showing the interaction (A). duration vs. solid: liquid using SSF (grain) (B). duration vs. solid: liquid using SmF (grain), (C) duration vs. solid: liquid using SSF (bran), (D). Duration vs temperature using SSF (grain). [Note: Grain PSD 1.18–0.6 mm and Bran PSD <0.6–0.3 mm were used; solid: liquid (%) was calculated as (w/v) ratio].

points for moisture absorption [40]. Initial total reducing sugar (TRS) and free amino nitrogen (FAN) concentrations increased with decreasing particle size, with the finest grain fraction (<0.3 mm) yielding the highest TRS (315.87 mg/L) and the finest bran fraction (<0.3 mm) producing the highest FAN (3.68 mg/L).

In general, bran fractions exhibited lower TRS but higher FAN (4.01–5.39 mg/L) than grain (2.97–3.88 mg/L), highlighting their protein-rich composition. Sorghum flour (SF) and wheat flour (WF) controls showed higher TRS (289.31 mg/L and 354.31 mg/L, respectively) and FAN (7.87 mg/L and 18.56 mg/L, respectively) values than

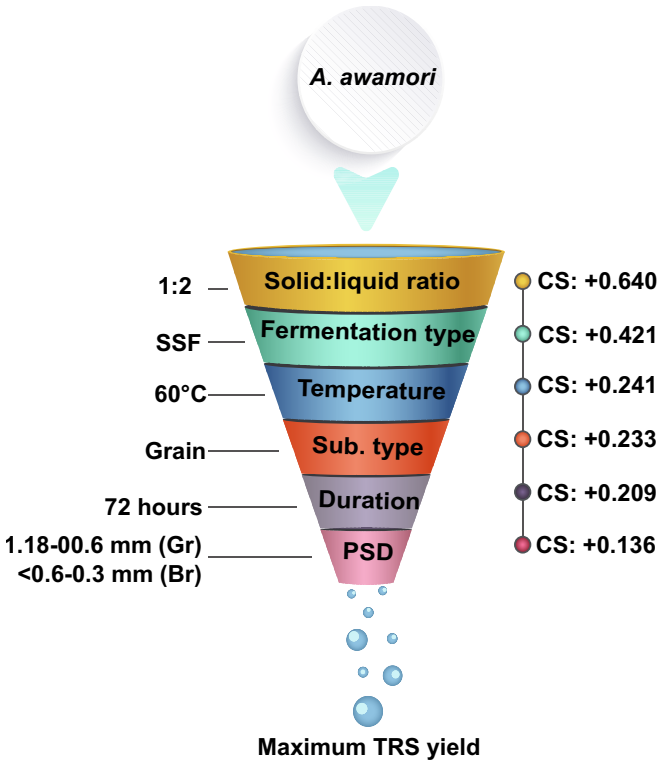

Fig. 11. Parameter optimization and arrangement based on correlation score. [CS: correlation score, Sub: substrate, PSD: particle size distribution, Gr: grain, and Br: Bran].

sorghum samples due to the presence of 0.2 % additional sugar and protein in commercial flour. The substrate-to-liquid ratio for the initial estimation of total reducing sugars (TRS) and free amino nitrogen (FAN) was maintained at 1:5 (w/v). Samples were prepared using the same protocol as those utilized for solid-state fermentation (SSF) and submerged fermentation (SmF) but without the addition of fungal inoculum. Total starch (TS) content in the sorghum grain and bran samples was determined to be 66.04 % and 39.41 %, respectively, and in the control samples, SF contained 69.06 %, while WF had 71.36 %. This approach ensured that the baseline physicochemical properties of the substrate were accurately determined prior to fermentation. The initial physicochemical characteristics of the sorghum samples, categorized by different particle sizes, are comprehensively summarized in Table 1. This analysis provides a foundational understanding of the substrate's properties, which is critical for interpreting the subsequent fermentation outcomes.

3.2. Critical insights for SSF

This study also investigated the deviation of pH, moisture content, and water during nine (09) days of solid-state fermentation (SSF) using *A. awamori*. For SSF media preparation, moisture content was adjusted to 75 % as per Eq. (5) and then sterilized using an autoclave as mentioned in Section 2.5. However, due to water evaporation during autoclaving, the moisture content has changed. Water loss was measured and adjusted with sterile water before inoculation with *A. awamori,* but during fungal fermentation (Day 1 to Day 9), pH and moisture content were only monitored without further adjustments. pH was measured every 24 h intervals using a mixture of 1 g of sample and 5 mL of water. Fig. 3 represents the pH fluctuation during SSF. Particle size has a significant influence on pH deviation, moisture retention, and water loss during solid-state fermentation (SSF) with *Aspergillus awamori*. Larger particles (>1.18 mm) exhibited the highest pH deviation (2.22), indicating greater acid production, while smaller particles (<0.3 mm) showed lower deviation (1.15–1.96), suggesting better pH stability. The higher metabolic activity of *A. awamori* results in a lower pH due to increased production of organic acids, such as citric acid. However, more fungal growth and metabolic activity generally result in a more significant pH decrease [41]. This suggests that a larger particle size may be more favourable for fungal growth and metabolism [42]. More significant pH fluctuations were observed in wheat flour, where the pH dropped from 6.76 to 3.34 within 48 h and again increased to 6.26 at 168 h. In contrast, sorghum flour and bran exhibited relatively stable pH fluctuations (±2.5) over a nine-day (09) period.

Filamentous fungi (i.e. *A. awamori, A. oryzae*) grow well at a pH range of 3.8 to 6.0 [43]. The critical insights within this range, as reported in Table 2, indicate no adverse effects on the feasibility of a sorghum-based biorefinery. Moisture loss was higher in larger grain and bran particles (5.42 %–5.89 %), while finer particles retained moisture (4.13 %–4.63 %) more effectively during fungal solid-state fermentation. Coarser particles (>1.18 mm) maintained higher moisture levels (~75 % on Day 1, ~69 % on Day 9) compared to finer particles (<0.3 mm), which exhibited greater moisture reduction (75 % on Day 1 to 71 % on Day 9).

Additionally, water loss during autoclaving was lower for smaller particles (0.768 g in grain <0.3 mm) compared to larger ones (1.159 g in grain <0.6–0.3 mm). Large particle sizes of both grain and bran exhibited greater pH deviation, moisture content variation, and water loss after autoclaving compared to smaller particles. Although larger particles posed more technological challenges, they also resulted in higher TRS and enzyme production. However, these challenges did not significantly impact overall TRS production, as discussed in the following sections on optimization.

3.3. Trend analysis for optimum particle size and culture duration

Trend analysis was conducted using *Aspergillus awamori* under solid-state fermentation (SSF) with an initial moisture content of 75 %. The primary objective was to determine the optimum duration for SSF and PSD for maximizing TRS, FAN, and α-amylase production. Sorghum grain and bran were used as the sole carbon and nitrogen sources, without the addition of any chemical additives or commercial media. Fermentation was conducted at 30 °C for nine (09) days, followed by saccharification at 30 °C for 60 min with a solid-to-liquid ratio of 1:20 and agitation at 180 rpm. The results, presented in Fig. 4, demonstrate the dynamic trends in TRS, FAN, and α-amylase production over the fermentation period.

The maximum peak for each particle size was observed at 96 h for all three trends (Fig. 4A, B, and C), where PSD (>0.6 mm) for both grain and bran showed a sharp peak followed by a decline after 96 h, while smaller particles (<0.3 mm) exhibited a more parallel pattern. Grain particles (1.18–0.6 mm) yielded the maximum TRS (14.51 g/L), while smaller particles (<0.3 mm) exhibited a lower TRS production. TRS production ranged from 10.50 to 14.51 g/L for grain and 4.37–9.75 g/L for bran (Fig. 4A). Fig. 4B illustrated the trend analysis of free amino nitrogen (FAN) production, showing comparable yields from grain and bran, with peak values of 35.19 mg/L and 34.87 mg/L, respectively, at 96 h. No significant differences were observed in FAN production with respect to particle size distribution (PSD). However, bran emerged as a slightly more favourable substrate for FAN production, potentially due to its higher protein content or enhanced nutrient accessibility [44]. Approximately 25 % of sorghum bran was recovered from sorghum seed, which can be valorized in a top-down biorefinery model by producing free amino nitrogen (FAN) for biofuel production as a nitrogen source and also enhancing its protein content through fungal processing for use as high-quality cattle feed, thereby advancing zero-waste objectives.

Fig. 4C illustrates the α-amylase activity produced by *A. awamori* using sorghum grain and bran as substrates under SSF. Similar to TRS and FAN production trends, maximum α-amylase activity was observed

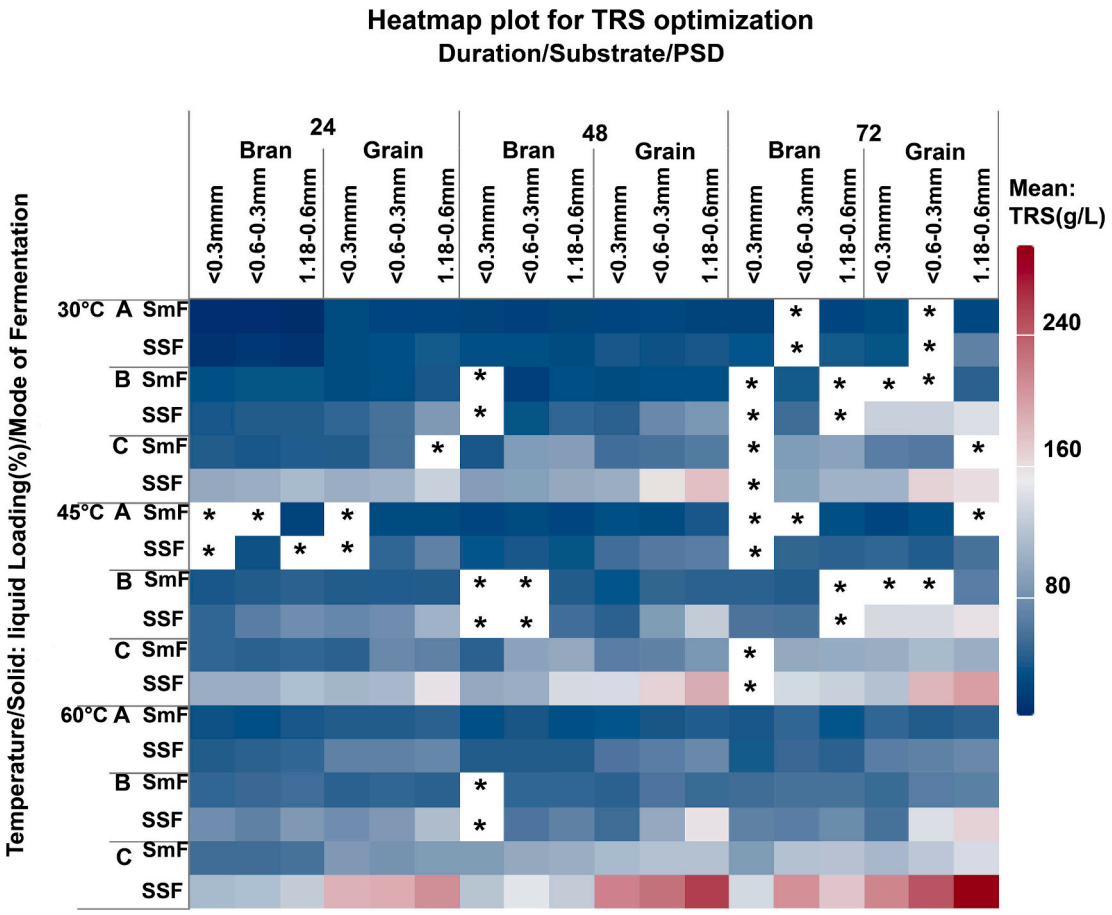

Fig. 12. Heatmap plot for parameter optimization and effects for TRS yield [*: Data not available; A: 1:10 (w/v); B: 1:4 (w/v); and C: 1:2 (w/v), PSD: particle size distribution].

Table 1
Initial characterization of Sorghum grain and bran.

Particle size distribution (PSD)	Bulk density (ρ_b) (g/cm^3)	Particle density (ρ_p) (g/cm^3)	Porosity# (Ɛ) (%)	Tortuosity# (τ) (dimensionless)	Initial moisture content# (%)	Initial TRS (mg/L)	Initial FAN (mg/L)	Total Starch (%)
Grain >1.18 mm	0.846 ± 0.002	1.457 ± 0.001	41.89 ± 0.08	0.393 ± 0.001	6.11 ± 0.02	122.01 ± 3.24	2.97 ± 0.08	Gr*
Grain 1.18–0.6 mm	0.854 ± 0.003	1.459 ± 0.001	41.47 ± 0.06	0.394 ± 0.001	4.72 ± 0.05	190.76 ± 3.21	3.74 ± 0.14	Gr*
Grain <0.6–0.3 mm	0.756 ± 0.001	1.459 ± 0.001	48.18 ± 0.11	0.379 ± 0.001	8.27 ± 0.03	313.72 ± 4.98	3.88 ± 0.24	Gr*
Grain <0.3 mm	0.486 ± 0.004	1.466 ± 0.004	66.85 ± 1.02	0.349 ± 0.002	8.16 ± 0.03	315.87 ± 5.15	3.68 ± 0.31	66.04
Bran 1.18–0.6 mm	0.553 ± 0.002	1.418 ± 0.001	61.01 ± 1.06	0.358 ± 0.001	4.74 ± 0.04	186.98 ± 4.66	4.01 ± 0.22	Br*
Bran <0.6–0.3 mm	0.402 ± 0.003	1.402 ± 0.002	71.3 ± 1.58	0.344 ± 0.001	6.79 ± 0.04	198.22 ± 2.12	5.27 ± 1.04	Br*
Bran <0.3 mm	0.366 ± 0.001	1.430 ± 0.002	74.4 ± 1.03	0.341 ± 0.001	8.51 ± 0.05	205.98 ± 2.45	5.39 ± 0.98	39.41
Sorghum flour, SF (Control)	0.536 ± 0.002	1.447 ± 0.001	62.95 ± 0.09	0.355 ± 0.002	7.15 ± 0.12	289.31 ± 3.11	7.87 ± 1.24	69.06
Wheat flour, WF (Control)	0.482 ± 0.002	1.456 ± 0.002	66.86 ± 0.05	0.349 ± 0.001	11.31 ± 0.23	354.31 ± 4.22	18.56 ± 2.1	71.36

Gr*: assumed to be the same percentage as in Grain<0.3 mm (66.04 %); Br*: assumed to be the same percentage as in Bran <0.3 mm (39.41 %); #: denotes statistical significant correlation with PSD at the 0.05 level (2-tailed); PSD vs. ρ_b: Pearson correlation (PC) = 0.743 and $P = 0.056$; PSD vs. ρ_p: PC = 0.072 and $P = 0.879$; PSD vs. Ɛ: PC = 0.761 and $P = 0.047$; PSD vs. T: PC =0.767 and $P = 0.044$; PSD vs. moisture contents: PC = 0.781 and $P = 0.038$. [Coefficient of variation for TRS: 2.014 % and FAN: 3.932 %].

at 96 h for grain with a particle size of 1.18–0.6 mm (22.83 U/mL). In contrast, smaller particles (<0.3 mm) exhibited a gradual increase in activity, reaching peak levels between 168 and 192 h for both grain and bran. Fig. 4C further demonstrated that sorghum grain was a more suitable substrate for α-amylase production than bran, with medium-sized particles (0.6–1.18 mm) being more effective. Control sorghum flour (SF) exhibited the highest TRS (17.10 g/L) and α-amylase activity (28.79 U/mL), while control wheat flour (WF) resulted in the highest FAN production (60.98 mg/L). This study provides key insights into the influence of particle size on SSF, such as: a). medium-sized particles (0.6–1.18 mm) for both grain and bran were optimal for fungal fermentation and yielded the highest TRS, FAN, and α-amylase

Table 2
Critical insights of solid-state fermentation (SSF).

Particle Size Distribution	pH			Moisture content (SSF, 30 °C)			Water loss
Bran and Grain	Initial pH (Day 0)	Final pH (Day 9)	pH deviation	Initial day moisture content (Day 0, %)	Final day moisture (Day 9, %)	Deviation	Water loss after autoclaving* (mL)
Grain >1.18 mm	5.58	3.36	2.22 (−)	74.59	68.70	5.89 (−)	0.945
Grain<1.18–0.6 mm	5.77	4.51	1.26 (−)	75.24	71.83	3.41 (−)	1.13
Grain<0.6–0.3 mm	5.69	4.48	1.21 (−)	74.05	69.26	4.79 (−)	1.159
Grain<0.3 mm	5.66	4.51	1.15 (−)	74.47	70.34	4.13 (−)	0.768
Bran 1.18–0.6 mm	5.43	3.63	1.80 (−)	75.36	69.94	5.42 (−)	1.07
Bran<0.6–0.3 mm	5.49	3.62	1.87 (−)	74.33	69.86	4.47(−)	1.06
Bran <0.3 mm	5.66	3.70	1.96 (−)	75.84	71.21	4.63 (−)	0.92
SF (Control)	6.66	4.74	1.92 (−)	74.43	71.43	3.06 (−)	1.18
WF (Control)	6.76	6.18	0.58 (−)	76.24	71.59	4.65 (−)	1.103

(−): indicates value decrease; (*): Autoclave conditions: 20 min at 121 °C at 15 psi, 1 mL equivalent 1 g. [Note: Fermentation: SSF; Fungal strain: *A. awamori*].

production, b). grain served as the superior substrate for TRS and α-amylase production, whereas bran was more suitable for FAN production. Although very fine particles offer a larger microbial surface area, excessive substrate accumulation hinders aeration, causing inadequate growth of microorganisms. Conversely, too large particles improve aeration efficiency but provide a small surface area for microbial action, resulting in lower growth of microorganisms [6]. Based on the trend analysis, five (05) days or 120 h of fermentation period was selected for further studies, as discussed in the following Section 3.4.

3.4. Comparative analysis of SSF and SmF for TRS and enzyme production

Saccharification for comparative analysis was conducted using a 5-day fungal culture, with a 75 % moisture content for SSF and 10 % for SmF. The process was carried out at 60 °C for 60 min, with a solid-to-liquid ratio of 1:8 for SSF. Fig. 5 presents a comparative analysis of solid-state fermentation (SSF) and submerged fermentation (SmF) for total reducing sugar (TRS) and enzyme production (α-amylase, glucoamylase, and protease) using *Aspergillus awamori*, based on different particle sizes of sorghum grain and bran. SSF consistently exhibited a significantly higher yield of TRS, α-amylase, glucoamylase, and protease than SmF across all particle sizes. The highest TRS yield (37.98 g/L) was obtained from grain with a particle size of 1.18–0.6 mm under SSF, whereas the lowest TRS yield (10.33 g/L) was observed from bran <0.3 mm using the same method. Comparative analysis revealed that grain substrates (ranging from 11.53 to 37.98 g/L) were significantly higher than bran substrates (ranging from 10.87 to 13.01 g/L) across both SSF and SmF. Notably, SSF resulted in a 3.3-fold higher TRS yield compared to SmF, emphasizing its superior efficiency in sugar production from sorghum substrates (Fig. 5A).

Fig. 5B depicts α-amylase activity, following a pattern like total reducing sugar (TRS) production, where solid-state fermentation (SSF) consistently exhibited higher enzyme yields than submerged fermentation (SmF) for all particle sizes. The highest α-amylase activity (109.55 U/mL) was recorded for SSF using grain with a particle size of 1.18–0.6 mm, while the second-highest activity (82.1 U/mL) was observed from bran with a particle size of <0.6–0.3 mm. The enzyme activity ranged from 50.14 to 109.55 U/mL in SSF and from 21.39 to 33.04 U/mL in SmF. Remarkably, SSF resulted in a 3.32-fold increase in α-amylase activity compared to SmF when using the same grain particle size (1.18–0.6 mm).

Fig. 5C illustrates glucoamylase activity, where solid-state fermentation (SSF) showed significantly higher enzyme production compared to submerged fermentation (SmF) throughout different particle sizes. The highest glucoamylase activity (2.31 U/mL) was observed in SSF using grain with a particle size of 1.18–0.6 mm, while the average enzyme production was 1.6 U/mL in SSF and 0.6 U/mL in SmF, indicating a 2.6-fold higher yield in SSF compared to SmF. Although grain demonstrated superior suitability for glucoamylase production, bran also proved to be an effective substrate, as evidenced by bran with a particle size of <0.6–0.3 mm exhibiting significant enzyme activity (1.64 U/mL) under SSF. In contrast, under SmF conditions, the highest glucoamylase activity (1.46 U/mL) was recorded for bran with a particle size of <0.3 mm, surpassing that of grain. These findings highlight the influence of substrate morphology on enzymatic efficiency, reinforcing the advantage of SSF for enhanced glucoamylase production.

Fig. 5D depicts protease production using *Aspergillus awamori*, which followed a distinct pattern compared to TRS, α-amylase, and glucoamylase activity. The highest protease activity (98.6 U/g, dry basis) was observed in SSF using bran with a particle size of <0.6–0.3 mm, followed by 84.61 U/g in bran with a particle size of 1.18–0.6 mm, confirming that SSF is the most effective fermentation mode for protease production. The higher protease yield in bran is attributed to its high protein content, which also contributed to increased free amino nitrogen (FAN) production. A comparative trend analysis of FAN (Fig. 4B) and protease activity (Fig. 5D) exhibited similar patterns, validating the experimental accuracy. In contrast, the high starch content in grain led to greater TRS, α-amylase, and glucoamylase production. These findings provide valuable insights for optimizing biorefinery processes, demonstrating that SSF is the optimal fermentation mode for TRS and enzyme production. Grain is the most suitable substrate for TRS, α-amylase, and glucoamylase, while bran is preferable for FAN and protease production.

Fig. 6 presents a comparative analysis similar to Fig. 5, but using *A. oryzae* instead of *A. awamori*, focusing on TRS and protease production. The highest TRS yield (20.69 g/L) was observed in SSF using grain with a particle size of 1.18–0.6 mm, following a pattern like *A. awamori,* but the TRS production ability of *A. oryzae* was 1.84 times lower than *A. awamori* under identical conditions, without parameter optimization or chemical supplementation (Fig. 6A). In contrast, protease production demonstrated a remarkable shift, with *A. oryzae* exhibiting an exceptional yield of 847.09 U/g (dry basis) from sorghum bran, which is 8.6-fold higher than *A. awamori* (Fig: 6B). This substantial increase in protease production under SSF conditions highlights the potential of sorghum bran, a by-product comprising 25 % of sorghum seed, as a valuable substrate for enzyme production.

Fig. 6B also elucidated that sorghum bran was significantly better than wheat flour for producing protease. This enhanced activity can be attributed to the superior nutritional composition of sorghum bran, particularly its higher protein (8.9–11.2 %), fiber (1.4–2.7 %), and phenolic content (175.75 mg/100 g), which are known to stimulate fungal metabolism and enzyme biosynthesis [6,45,46]. Chutmanop, et al. [47] achieved 920 U/g protease from wheat barn using *A. oryzae* under SSF after process optimization. Meena, et al. [48] reported 582.25 ± 9.2 U/mL protease production from wheat bran using bacterium *Pseudomonas aeruginosa* under solid-state fermentation, while Balachandran, et al. [49] achieved 397.09 U/mL using *Bacillus halodurans* with the same substrate and fermentation method. The significant

protease yield from *A. oryzae* suggests its potential as a game-changer for the sorghum-based biofuel and enzyme production industries (Fig. 6B). *A. oryzae* produces enzyme complexes rich in proteolytic enzymes, which aligns with the findings of Dorado, et al. [14] on cereal-based biorefinery development.

Additionally, Wang, et al. [50] reported that *A. awamori* is an effective producer of amylase and glucoamylase, whereas *A. oryzae* efficiently converts protein into free amino nitrogen (FAN), supporting its role in nitrogen source generation. SSF remains the superior fermentation technique, not only due to its high product yield but also because of lower water and energy consumption and greater cost-effectiveness, making it ideal for agro-industrial by-product utilization [51–53]. SSF is particularly suitable for filamentous fungi, as it provides a natural growth environment, whereas SmF represents an unnatural environment for these microorganisms [54].

This study primarily focused on TRS production and its optimization, as TRS is the key precursor for bioethanol production, where an increase in TRS enhances bioethanol yield. The subsequent section details the optimization of TRS production, employing grain with a particle size of 1.18–0.6 mm, SSF, and a five-day culture of *A. awamori*. Saccharification was optimized by fine-tuning of temperature, duration, and solid-liquid ratio to maximize TRS yield.

3.5. Process optimization

3.5.1. Comparison of saccharification efficiency: Fungal-treated vs. commercial enzyme-treated samples

Samples were prepared as described in Section 2.8.1, maintaining a solid-to-liquid ratio of 1:2 for saccharification.

Table 3 shows the in-situ enzyme cocktail, derived from 2.5 g fungal mash using *A. awamori*, contained α-amylase (982 ± 9.45 U/mL), glucoamylase (10.93 ± 0.58 U/mL), and protease (168.05 ± 4.38 U/g, db), while the prepared commercial enzyme cocktail (CEC) comprised α-amylase (1050 U/mg), glucoamylase (13 U/mL), and protease (500 U/g). Fig. 7 presents the saccharification efficiency achieved using both enzyme cocktail (in situ and commercial) treatments. For this study, grain with a particle size distribution (PSD) of 1.18–0.6 mm and bran with PSD <0.6–0.3 mm were selected. *Aspergillus awamori* was used as the fungal strain, and solid-state fermentation (SSF) was employed, as prior experiments (Sections 3.2 and 3.3) indicated that these conditions were optimal for total reducing sugar (TRS) production. The highest TRS yield was observed at 48 h of saccharification at 60 °C, with 144.83 g/L from grain treated with *A. awamori* fungal mash and 122.11 g/L from grain treated with the commercial enzyme.

Notably, saccharification of commercial sorghum flour (SF) using fungal mash resulted in a significantly higher TRS yield (194.14 g/L) compared to commercial enzyme treatment (90.76 g/L), demonstrating more than twice the efficiency of the in-situ enzyme cocktail. Furthermore, *A. awamori* exhibited superior starch saccharification ability compared to *A. oryzae*, and grain proved to be the most effective substrate for TRS production, aligning with findings from previous experiments (Sections 3.4 and 3.5). The saccharification duration had no significant impact on yield, with only an 8.12 g/L increase observed from 24 h to 96 h for grain treated with commercial enzyme. In contrast, for the fungal mash, the variation in TRS yield remained minimal (±1.24 g/L) between 48 h and 96 h. From an economic and industrial perspective, this study suggests that 48 h of saccharification appears to be the most efficient and feasible duration for achieving optimal TRS production.

The total reducing sugar (TRS) production rate using fungal mash was notably higher than that observed with commercially available enzyme complexes. This result aligns with the findings by Kiran, et al. [55], who demonstrated the superior efficiency of fungal mash in hydrolyzing mixed food waste substrates during enzymatic pretreatment. Interestingly, despite the higher concentration of commercial enzyme complexes compared to the in-situ enzyme complex (Table 3), saccharification activity was unexpectedly greater with the in-situ enzyme complex. This phenomenon highlights the synergistic enzymatic activities inherent to the fungal mash. Previous studies Koutinas, et al. [15] and López, et al. [56] stated *Aspergillus awamori* as a prolific producer of glucoamylases.

Furthermore, it synthesizes a diverse array of hydrolytic enzymes, including amylases, proteases, cellulases, and xylanases, particularly when cultivated on complex substrates through solid-state fermentation (SSF). López, et al. [56] further reported that fermented solids derived from the SSF of babassu cake with *A. awamori* exhibited substantial enzymatic activities—not only proteases and xylanases but also cellulases alongside amylases. This versatile enzymatic profile enables *A. awamori* to produce a robust enzyme complex, outperforming selectively prepared commercial enzyme mixtures in starch hydrolysis. Kiran and Liu [57] documented that fungal mash contains additional carbohydrases such as α-glucosidases, β-amylases, β-glucanases, pullulanase, cellulases, xylanases, and hemicellulases, complementing its glucoamylase activity.

Fourier Transform Infrared (FTIR) spectroscopy was used to observe qualitative changes in the chemical structure of sorghum grain (particle size 1.18–0.6 mm) by analysing the positions and shapes of key absorption bands between untreated and enzyme-treated samples. In Fig. 8, spectrum shows some characteristic band in the following regions, 3300 cm^{-1}: the O—H stretching of starch, 2940 cm^{-1}: stretching vibration of CH and CH_2 of starch, 1500 cm^{-1}: characteristic of aromatic C—C bonds of starch, and 1018–1072 cm^{-1}: deformation of C—H type bond of starch molecule [58–60]. A slight smoothing in the 1080–1265 cm^{-1} region, particularly in enzyme-treated and in situ enzyme-treated (Fig. 8) samples, suggests minor disruptions to polysaccharide structures due to enzymatic hydrolysis. These spectral changes, observed in both commercial and in situ fungal enzyme-treated samples, indicate partial cleavage of glycosidic bonds within the starch molecules of sorghum grains.

FTIR analysis was performed as a qualitative tool to observe spectral pattern changes between untreated (A. black line), commercial enzyme-treated (B. blue line), and in situ enzyme-treated (C. pink line) sorghum

Table 3
Enzyme activity of in situ-produced and commercial enzymes.

Fungal strain	2.5 g fungal mash from SSF (In-situ enzyme complex)							Commercial enzyme complex		
	Fermentation mode	Substrate	TRS[a]	FAN[a]	α-amylase[b]	Glucoamylase[b]	Protease[b]	α-amylase	Glucoamylase	Protease
			g/L	mg/L	U/mL	U/mL	U/g (75 % mash, db)	U/mg	U/mL	U/g
A. awamori	SSF	Grain	27.84 ± 0.18	31.66 ± 2.31	982.5 ± 7.45	10.925 ± 0.58	168.05 ± 4.35	1050	13	500
	SSF	Bran	1.73 ± 0.11	7.76 ± 0.36	489.8 ± 6.87	1.2 ± 0.032	85.75 ± 2.85			
A. oryzae	SSF	Grain	17.89 ± 0.27	11.57 ± 0.43	688.325 ± 6.21	3.575 ± 0.19	109.7 ± 3.97			
	SSF	Bran	1.07 ± 0.15	42.43 ± 1.12	262.525 ± 3.45	0.175 ± 0.02	634.38 ± 8.24			

[a] Estimated after 5 days fungal fermentation at 30 °C and saccharification duration 60 min at 60 °C.
[b] Estimated after 5 days fungal fermentation at 30 °C and saccharification duration 24 h at 60 °C, solid-liquid ratio: 1:2 (w/v)]. The number represents the average of triplicates ± standard deviation.

grain samples. The aim was not to draw quantitative conclusions on enzymatic digestibility but to validate treatment effects by comparing functional group profiles. The results showed that commercial and in situ enzyme-treated samples exhibited similar FTIR spectra, both differing from the untreated sample, suggesting that the enzyme produced in this study functions similarly to the commercial enzyme. Thus, FTIR served as a method validation step to confirm that enzymatic treatments induced comparable structural modifications in the substrate. Compared to commercial enzymes, the fungal mash offers significant economic advantages by eliminating the enzyme extraction step, as it reduces both operational costs and resource constraints, providing a cost-effective and sustainable alternative for industrial saccharification processes, as discussed in Section 3.6.1. These findings underscore the potential of fungal mash as a viable and efficient enzymatic solution for bioconversion applications.

3.5.2. Optimization of saccharification temperature and duration

Fig. 9A, illustrates the effect of saccharification duration and temperature on TRS yield, using grain (1.18–0.6 mm) as a substrate with a solid-liquid ratio of 1:2. Saccharification was performed at four different temperatures (30 °C, 40 °C, 50 °C, and 60 °C) over durations ranging from 24 to 72 h. A strong correlation was observed between TRS yield and temperature (Pearson correlation, $r = +0.241$, $p < 0.05$) as well as saccharification duration (Pearson correlation, $r = +0.209$, $p < 0.05$). These findings indicate that increasing temperature and duration have a positive influence on TRS yield (details of correlation and heatmap analysis are discussed in Section 3.6). The maximum TRS yield (289.68 ± 3.48 g/L) was achieved at 60 °C after 48 h, while the minimum yield (42.21 ± 2.18 g/L) was recorded at 30 °C after 24 h. Notably, a temperature increases from 30 °C to 60 °C resulted in a 5.4-fold enhancement in TRS yield. Fig. 9A, also showed almost similar TRS production at 48 h (289.68 ± 3.48 g/L) and 72 h (287.51 ± 4.37 g/L) at 60 °C, and no statistically significant difference was observed. Due to this unexpected similarity, additional temperature and duration experiments were conducted, as shown in Fig. 9B and Fig. 10D, for accuracy and method validation.

However, saccharification duration showed a weaker influence on yield with duration; at 60 °C, TRS yield increased by only 1.13-fold (13.63 %) from 24 h to 48 h (254.93 g/L to 289.68 g/L). Remarkably, saccharification temperatures above 55 °C enhanced starch-to-glucose conversion efficiency, according to the maximal activity of hydrolytic enzymes such as glucoamylase, protease, and xylanase [61]. Additionally, fungal autolysis was observed at 60 °C, contributing to an increase in TRS release [14,61]. Dorado, et al. [14] reported that in temperatures exceeding 55 °C, starch-to-glucose conversion efficiency reached its maximum, coinciding with optimal enzyme activity and fungal autolysis. Enzymes such as glucoamylase, protease, and xylanase exhibited peak hydrolytic activity at temperatures above 55 °C, with fungal autolysis significantly contributing to the increased TRS yield at 60 °C. This enzymatic synergy was pivotal in maximizing TRS production [14,61]. As reported in Dorado, et al. [14], simultaneous starch and protein hydrolysis, combined with fungal autolysis, was observed within 30 h, explaining the high TRS yield at 48 h compared to 24 h. This aligns with the observed pattern in this study, where prolonged saccharification yielded diminishing returns beyond the 48-h mark, likely due to substrate depletion and enzyme inactivation.

3.5.3. Effect of solid: liquid loading

Based on findings from prior experiments, the optimal parameters-SSF using *A. awamori*, sorghum grain particle size of 1.18–0.6 mm, and saccharification temperature of 60 °C were considered for the solid: liquid loading experiment. The maximum total reducing sugar (TRS) yield was observed when the solid-liquid ratio was maintained at 1:2. Under these conditions, the maximum TRS yield recorded was 304.83 g/L after 72 h, with the second-highest yield of 290.76 g/L achieved after 48 h (Fig. 9B). This is a significant improvement compared to Barcelos, et al. [17], who achieved a glucose concentration of 193.4 g/L using commercial enzymes at a solid loading of 1:3 (w/v). It is important to note that a solid-liquid ratio of 1:1 was not feasible, as all liquid was absorbed by the grain powder, making liquid extraction impossible. Therefore, a 1:2 (solid: liquid, w/v) ratio was maintained to achieve the maximum substrate concentration for this study. After comprehensive optimization of key process parameters, it was concluded that a maximum TRS yield of 304.83 g/L could be achieved from 500 g/L of sorghum grain under the following conditions: solid-state fermentation (SSF) using *Aspergillus awamori*, a particle size of 1.18–0.6 mm, a fermentation duration of five days, saccharification at 60 °C for 72 h, a solid-liquid ratio of 1:2, and an agitation rate of 180 rpm (Fig. 9B).

3.6. Method validation

Residual diagnostics (normal probability plot, histogram, Versus Fits, and Versus Order) were performed to evaluate the assumptions of the regression model for total reducing sugar (TRS) production (See Supplementary Fig.S5). The normal probability plot and histogram confirm that the residuals are approximately normally distributed, satisfying the assumption of normality. The Versus Fits plot indicates that the residuals are randomly scattered, with no discernible patterns, ensuring constant variance. Additionally, the Versus Order plot demonstrates the independence of residuals with no observable trends. These results validate the model's appropriateness for describing TRS production and its predictive accuracy. The experimental data obtained from practical trials were analyzed using statistical modelling to evaluate validity and accuracy.

Model Summary:

S	R^2	R^2(adj)	R^2(pred)
18.3938	94.12 %	92.30 %	86.95 %

The model demonstrates a strong fit to the data, with an R^2 of 94.12 %, indicating that 94.12 % of the variability in TRS production is explained by the predictors. The adjusted R^2 (92.30 %) and predicted R^2 (86.95 %) suggest good model generalizability with minimal overfitting. Contour plots were generated to visualize these relationships, providing insights into the combined influence of the parameters (Fig. 10 and supplementary Table S1). The contours plot in Fig. 10 illustrates the interaction effects of saccharification duration, solid: liquid, and temperature on total reducing sugar (TRS) yield (g/L) under different experimental conditions.

Fig. 10A represents the interaction between solid: liquid and saccharification duration at a fixed temperature of 60 °C under solid-state fermentation using *A. awamori* and grain (PSD 1.18–0.6 mm) as a substrate. At point A, solid: liquid ratio of 11.62 % and a duration of 26.24 h resulted in a TRS yield of 86.24 g/L. Increasing the solid: liquid ratio to 31.89 % and duration to 54.23 h enhanced the yield to 183.52 g/L. The TRS yield of 251.57 g/L was observed at point C, with a solid: liquid ratio of 47.88 % and a duration of 69.18 h. Solid: liquid loading and saccharification duration exhibited a positive correlation, as summarized in Supplementary Table S1. A similar trend was observed in Fig. 10B and C, where the process conditions varied, with SmF replacing SSF and bran used as the substrate instead of grain, respectively. Fig. 10B demonstrates that the highest TRS yield of 101.48 g/L was achieved using SmF at point C under conditions of 47.97 % solid: liquid ratio and a saccharification duration of 70.06 h. However, this yield is substantially lower compared to Fig. 10A, indicating that TRS production under identical conditions is approximately 2.5 times higher when employing the SSF method. This finding aligns with the trend reported in Section 3.3, where SSF was shown to be the superior fermentation strategy over SmF for TRS production.

Furthermore, when sorghum bran was used as the substrate under the same conditions as in Fig. 10A, the TRS yield was estimated at

around 75.74 g/L at point A (solid: liquid ratio 48.89 %, and saccharification duration of 46.15 h) of Fig. 10C. These findings indicate that sorghum bran is not a suitable substrate for efficient TRS production. Fig. 10D depicts the effect of duration and temperature on TRS yield using grain as the substrate under SSF at 50 °C. Point A of Fig. 10D represents a temperature of 36.87 °C and a saccharification duration of 48.07 h, yielding 160.07 g/L TRS. In contrast, at point B, where the temperature was increased to 57.89 °C while maintaining the same saccharification duration, the TRS yield significantly increased to 226.89 g/L. This corresponds to a 41.7 % enhancement in yield with a temperature increase of 21.02 °C, highlighting temperature as a critical parameter influencing TRS production. These findings underscore that, compared to duration, temperature plays a more decisive role in optimizing saccharification efficiency.

3.6.1. Correlation of TRS production with process parameters

Pearson correlation analysis and dot matrix plotting were employed to elucidate the most impactful parameter contributing to the fine-tuning and maximization of total reducing sugar (TRS) yield. A *p*-value of less than 0.05 was considered statistically significant, indicating a strong association between the respective parameters and TRS yield. The strongest correlation was observed between TRS yield and solid: liquid ratio (correlation score, CS = +0.644, 95 % confidence interval, CI: 0.566–0.703, $p < 0.05$), followed by TRS yield and mode of fermentation (CS = +0.421, 95 % CI: 0.321–0.339, $p < 0.05$). In contrast, TRS yield exhibited the weakest correlation with particle size distribution (CS = +0.136, 95 % CI: 0.21–0.248, $p = 0.021$) compared to the other parameters.

Scanning electron microscopy (SEM) revealed that large particles (>1.18 mm) exhibited smooth surfaces and high *v*oid spaces (Fig. 1A), leading to reduced surface area and weak fungal attachment. Particles in the 1.18–0.6 mm range (Fig. 1B) showed fractured surfaces with fine particles adhered to larger ones, enhancing surface area and porosity, thus promoting oxygen transfer, fungal colonization, and product yield, making this the most effective size range for SSF. Conversely, very small particles (<0.3 mm, Fig. 1D) were highly compact with minimal *v*oid space, which, despite a higher surface area, hindered aeration and nutrient diffusion, resulting in reduced fungal activity and TRS yield. The presence of irregular cracked surfaces and microparticle adherence might be the cause of a non-linear relationship between particle size, fungal growth, and product formation. All correlations were statistically significant.

A correlation score (CS) analysis was conducted to *e*valuate the influence of six (06) key parameters on total reducing sugar (TRS) yield. Based on the correlation coefficients, the parameters were ranked in descending order of impact as follows: (1) solid: liquid ratio (CS = +0.640, 95 % CI: 0.566–0.703, $p < 0.05$), (2) fermentation type (CS = +0.421, 95 % CI: 0.321–0.339, $p < 0.05$, (3) saccharification temperature(CS = + 0.241, 95 % CI: 0.128–0.374, $p < 0.05$), (4) substrate type (CS = +0.233, 95 % CI: 0.120–0.339, $p < 0.05$), (5) duration (CS = + 0.209, 95 % CI: 0.095–0.317, $p < 0.021$), and (6) particle size distribution (CS = +0.136, 95 % CI: 0.21–0.248, $p < 0.05$). The sequential ranking is illustrated in Fig. 11 and supplementary Table S1, reflecting the relative strength of association between each parameter and TRS yield.

All key parameters influencing total reducing sugar (TRS) production were systematically analyzed and presented using a heatmap, illustrating the optimization of TRS yield as a function of saccharification duration, substrate type, particle size distribution (PSD), temperature, solid: liquid percentage, and fermentation mode (solid-state fermentation, SSF, vs. submerged fermentation, SmF). The heatmap provides a comprehensive visualization of TRS production dynamics during *Aspergillus awamori* fermentation, highlighting the interactive effects of these variables and identifying optimal conditions for maximizing TRS yield (Fig. 12).

A clear trend is observed in Fig. 12, where TRS yield increases with temperature, solid: liquid ratio, and duration. Notably, the highest TRS yield of ~250–260 g/L is achieved at 60 °C, 1:2 solid: liquid ratio, and 72 h of saccharification under SSF using grain (1.18–0.6 mm) as the substrate. Lower TRS yields are observed at 30 °C, regardless of fermentation mode and substrate, with a minimum yield of ~40–50 g/L at 1:10 (w/v) substrate loading after 24 h. A significant enhancement in TRS yield occurs at 45 °C, particularly with SSF at a 1:4 (w/v) and 1:2 (w/v) solid-to-liquid ratio, where *v*alues exceed 160 g/L, demonstrating a positive correlation between solid-to-liquid loading and saccharification efficiency. A transition from SmF to SSF results in a marked increase in TRS yield, particularly at 1:2(w/v) loading, where SmF exhibits lower efficiency, yielding ~100–120 g/L compared to SSF's peak >250 g/L. The effect of substrate type and PSD is also evident, with grain (1.18–0.6 mm) outperforming bran (<0.3 mm and 0.6–0.3 mm) in TRS production under identical conditions. The impact of duration is pronounced, with a 72-h saccharification period yielding up to ~304.83 g/L under optimized conditions, compared to ~290.76 g/L at 48 h and ~ 86.24 g/L at 24 h.

Fig. 12 also reveals that there is no significant increase in total reducing sugar (TRS) yield when extending the saccharification duration from 48 to 72 h at 60 °C with 1:2 (w/v) grain solid-liquid ratio, with TRS production stabilizing around 300 g/L. For industrial applications, prolonging the process beyond 48 h would increase additional costs, including electricity, machine operation, labor, and facility maintenance, without a substantial product yield. Thus, an optimized saccharification duration of 42 h is recommended to maximize efficiency while minimizing operational expenses. In addition, 100 % saccharification is achieved within the first 30 h, as mentioned by Dorado, et al. [14]. Sankar et al. [16] and Du ChenYu et al. [17] reported that SSF exhibits superior saccharification efficiency and higher yield compared to SmF, using rice straw and wheat bran as substrates, respectively. A high insoluble solid-liquid ratio enhances sugar production, thereby increasing ethanol yield [62]. However, excessive substrate concentration can reduce hydrolysis efficiency due to increased viscosity, leading to poor mixing and mass transfer limitations [63,64]. Therefore, optimizing substrate concentration and temperature is crucial for maximizing sugar production. Saccharification efficiency is significantly enhanced when using an enzyme complex rather than a single enzyme. Husin, et al. [65] reported that the use of amylase and cellulase mixture resulted in higher fermentable sugar production compared to the use of either amylase or cellulase alone. Table 4 presents a comparative analysis between previous studies and the current study, highlighting various saccharification processes and fungal hydrolysis methods applied to different substrates for total reducing sugar (TRS) production.

Barcelos, et al. [17] reported, sorghum grain hydrolyzed with commercial enzymes at 80 °C achieved the highest TRS yield of 193.4 g/L at a particle size of 0.5 mm, while fungal hydrolysis using *Phanerochaete chrysosporium* on sorghum grain stillage and sorghum husk yielded significantly lower TRS levels, with 19.74 g/100 g and 103.0 mg/g, respectively studied by Ren, et al. [18] and Waghmare, et al. [66]. In addition, Waghmare, et al. [66] demonstrated that enzymatic hydrolysis of untreated sorghum husk yielded 20.07 mg/g of reducing sugars, whereas biological pretreatment of the sorghum husk prior to enzymatic hydrolysis significantly enhanced the yield to 103.0 mg/g. These findings highlight the effectiveness of biological pretreatment in substantially improving total reducing sugar (TRS) production from sorghum husk compared to the untreated material. Wheat flour subjected to acid hydrolysis followed by *A. awamori* and *A. oryzae* fermentation yielded 140 g/L glucose, reported by Du, et al. [16]. Microwave-assisted pretreatment and acid hydrolysis of maize distillery stillage produced the lowest TRS yield of 10.40 mg/g, emphasizing the importance of optimized enzymatic hydrolysis for maximizing sugar recovery [67]. In another study by Makanjuola, et al. [31], a glucose yield of 193.5 mg/g and 38.7 g/L was obtained in a 2 L bioreactor after optimizing glucoamylase activity from sorghum (37.6 U/mL after 115 h), but the particle

Table 4
Comparative analysis of total reducing sugar (TRS) yield among previous studies and the present study.

Substrate	Saccharification process	Fungal treatment	PSD	Maximum Yield of TRS	References
Sorghum grain	**HT:** 80 °C for 30 min **Hydrolysis**: Commercial glucoamylase and α-amylase: 40 μL/g **Solid-liquid ratio:** 1:3 (w/v)	No	• 0.7 mm • 0.5 mm • 0.3 mm	193.4 g/L (0.5 mm)	Barcelos, et al. [17]
Grain stillage from liquor industry (Sorghum)	**HT:** Autoclave and microwave-assisted hydrothermal, **Hydrolysis:** commercial enzyme (fibrolytic enzymes cellulase:1×10^3 U/g, xylanase: 5×10^2 U/g, β-glucosidase: 1×10^2 U/g (Total vol: 150 U/g) **Temp:** 50 °C **Duration:** 72 h **Solid-liquid Ratio:** 1:15	*P. chrysosporium* **Duration**: 6 days, **Inc. size**:10 %, **Temp.**: 28 °C	0.2–0.25 mm	19.74 g/100 g	Ren, et al. [18]
Sorghum husk	In situ enzyme **Duration**: 48 h **Temp.**: 50 °C **RPM:** 110	*P. chrysosporium*, **Duration:** 8 days, **FT:** SmF **Temp.**: 30 °C **Chemical media**: (15 g sample 300 ml in Dubos medium)	<1 mm	103.0 mg/g	Waghmare, et al. [66]
Sorghum bran	**Commercial Enzyme**: (amylase: 250 U/g; amyloglycosidase: 250 U/g) **Duration:** 24 h **Temp.:** RT **Ch. Tr.**: DMSO and acid hydrolysis	No	–	61 g/L	El-Imam, et al. [19]
Wheat flour	**HT**: autoclave **Ch. Tr**: 2 M H_2SO_4 **Solid-liquid ratio**: 1:10	*A. awamori* (96 h) and *A. oryzae* (48 h)	0.5 mm	140 g/L (glucose)	Du, et al. [16]
Sorghum bran	**HT:** autoclave (15 min for 121 °C) **Hydrolysis:** in-situ produced glucoamylase **Duration**: 120 h **Temp.:** 55 °C **RPM:** 200 **Solid-liquid Ratio:** 1:3	*A. awamori* **Duration:** 2 days **Temp.:** 30 °C **FT:** SmF **Inc. size**: 10^7 spores/g **Nutrition:** 2.5 g/L yeast extract and commercial mineral solution	>1 mm	193.5 mg/g (glucose)	Makanjuola, et al. [31]
Maize distillery stillage	**HT:** microwave pretreatment (300 W, 54 PSI, 15 min), H_2SO_4 **Hydrolysis:** commercial enzyme, cellulase16 μL/g sample **Duration:** 24 h Temp.:50 °C **RPM:** 70 rpm **Ch. Tr.:** H_2SO_4	No	–	10.40 mg/g	Mikulski, et al. [67]
Sorghum grain	**HT:** autoclave (15 min for 121 °C) **Hydrolysis:** Fungal mash with in-situ enzyme complex **Duration**: 72 h **Temp.:** 60 °C **RPM:** 180 **Solid-liquid Ratio:** 1:2	*A. awamori* **Duration:** 5 days **Temp.:** 30 °C **FT:** SSF **Inc. size**: 10^6 spores/10 g	• >1.18 mm • 1.18–0.6 mm • <0.6–0.3 mm • <0.3 mm	304.87 g/L (609.74 mg/g, db) (PSD:1.18–0.6 mm)	This study
Sorghum bran	**HT:** autoclave (15 min for 121 °C) **Hydrolysis:** Fungal mash with in-situ enzyme complex **Duration**: 72 h **Temp.:** 60 °C **RPM:** 180 **Solid-liquid Ratio:** 1:2	*A. awamori* **Duration:** 5 days **Temp.:** 30 °C **FT:** SSF **Inc. size**: 10^6 spores/10 g	• 1.18–0.6 mm • <0.6–0.3 mm • <0.3 mm	156.98 g/L (313.96 mg/g,db) (PSD: <0.6–0.3 mm)	This study

[Note: HT: heat treatment; Temp.: Temperature; FT: fermentation type; Ch. Tr: chemical treatment; Inc.: inoculum].

size of sorghum was not investigated, and a commercial nitrogen source was added. Overall, previous studies mentioned in Table 4 have overlooked several critical aspects. Firstly, most research has relied on commercial enzymes and chemicals for starch saccharification, which significantly increases production costs. Secondly, no studies have systematically evaluated the impact of particle size fractionation on SSF and SmF using fungal strains. Thirdly, there is a lack of comparative analysis between sorghum grain and bran portions concerning their effectiveness in total reducing sugar (TRS) production. This study addresses these gaps and demonstrates optimized conditions that achieve the highest TRS yield reported to date, establishing a new benchmark in sorghum-based saccharification research.

In this study, sorghum grain with a particle size distribution of 1.08–0.6 mm yielded a maximum TRS concentration of 304.83 g/L (609.74 mg/g, dry weight basis), while sorghum bran achieved 160 g/L (313.96 mg/g, dry weight) through *A.awamori*-mediated solid-state fermentation (SSF) without the use of commercial enzymes, acid, or alkali pretreatment. Notably, this study achieved the highest total reducing sugar (TRS) yield reported to date from sorghum-based substrates (grain, bran, or husk), surpassing all previously published values (Table 4). This was achieved through integrated saccharification parameter optimization, precise particle size fractionation, and fungal fermentation, without the addition of any commercial enzymatic hydrolysis, thereby demonstrating a significant advancement in bioconversion efficiency. Moreover, saccharification efficiency (dry weight basis) was observed at 83.17 % for sorghum grain and 72.69 % for sorghum bran, whereas the control samples of sorghum flour (SF) and wheat flour (WF) exhibited saccharification yields of 81.55 % and 77.76 %, respectively using fungal mash with in-situ enzyme complex. Saccharification efficiency using *A. awamori* for grain and bran is listed in Supplementary Table S2. This study establishes a cost-effective and environmentally friendly saccharification process leveraging fungal fermentation within the sorghum biorefinery framework, presenting a sustainable and efficient alternative to conventional hydrolysis methods. Similarly, Dorado, et al. [15] reported significant hydrolysis yields using fungal fermentation with wheat as a substrate, further

supporting the efficacy of this approach. These findings confirm that higher temperature, prolonged saccharification, increased solid-liquid ratio, and the SSF mode significantly enhance TRS production, providing crucial insights into the optimization of fungal fermentation for industrial bioprocessing applications.

This study demonstrates a cost-effective and sustainable approach to starch-based biorefinery utilizing fungal fermentation. The current production cost (including materials, milling, labor, utilities, and maintenance) of sugar from starch ranges from $0.22 to $0.33/kg [68], while enzymatic hydrolysis alone requires $0.088–$0.11/kg of corn [69]. Although the additional sieving step to obtain specific particle sizes could slightly increase the refining cost of this process, the total production cost was assumed to be $0.27 per kilogram of sugar, while enzymatic hydrolysis, an average cost of $0.099 per kilogram, was used in Eq. (8) below-

$$\text{New production cost (NPC), USD/kg} = 0.27 - 0.099 = 0.171 \text{ USD/kg} \quad (8)$$

$$\text{Reduced production cost (\%)} = (0.099/0.27) \times 100 = 36\% \quad (9)$$

Further studies should estimate the exact cost of milling and sieving sorghum to enhance the accuracy of the cost analysis. The elimination of the commercial enzymatic hydrolysis process enables a cost reduction of approximately 36 % in the proposed fungal saccharification method, resulting in a new production cost of $0.171/kg of sugar. Furthermore, optimizing just six (06) key parameters enabled 83.17 % saccharification efficiency, highlighting the potential for further process improvements. It provides critical insights into saccharification processes and parameter optimization to maximize total reducing sugar (TRS) yield. The findings establish optimized ranges for key parameters, which can serve as the foundation for future bioprocessing research and be applied in the development of a robust design of experiments (DoE). Beyond its economic advantages, this approach substantially reduces greenhouse gas emissions by eliminating incineration, high-temperature boiling, and the use of strong acids or bases for biomass pretreatment. This approach minimizes energy consumption and eliminates the generation of toxic byproducts, offering a more environmentally sustainable alternative to conventional biomass processing. It would establish a milestone framework for optimizing bioprocessing parameters, providing a scalable and sustainable solution for grain and lignocellulosic materials processing in the biorefinery industry.

4. Conclusion

This study presents a novel and cost-effective biorefinery process for producing reducing sugars from sorghum grain under optimized solid-state fermentation (SSF) conditions, achieving a remarkable TRS yield of 304.87 g/L (83.17 % saccharification efficiency) without pH and moisture content control. Solid-liquid ratio (CS = +0.644, 95 % CI: 0.566–0.703, $p < 0.05$) and mode of fermentation (CS = +0.421, 95 % CI: 0.321–0.521, $p < 0.05$) were identified as the key parameters most strongly associated with TRS production. Using *A. awamori* in solid-state fermentation (SSF), a particle size distribution (PSD) of >0.6 mm yielded 37.97 g/L of total reducing sugars (TRS), which was 1.7 times (76.1 %) higher than that obtained from finer particles (<0.3 mm, 21.56 g/L). This suggests that coarser particles are optimal for fungal fermentation and TRS production, as they provide better aeration, a higher surface area, and a stronger attachment surface, which are favourable for fungal growth under the given conditions. Conversely, when *A. oryzae* was used with sorghum bran (PSD of <0.6–0.3 mm), resulted in an 8.5-fold higher protease yield compared to sorghum grain of similar particle size under SSF. Notably, increasing the saccharification temperature from 30 °C to 60 °C led to a 5.4-fold enhancement of fermentable sugar production. The results highlight the suitability of *A. awamori* for α-amylase and TRS production using grain substrates, while *A. oryzae* and bran excel in FAN and protease production. This dual-purpose approach not only supports bioethanol and biofuel production but also generates residues rich in protein, suitable for cattle feed, thereby contributing to zero-waste and eco-friendly bioprocessing within a top-down biorefinery framework. The validated methodology provides accurate, sequential results and insights for further parameter tuning, paving the way toward achieving 100 % saccharification efficiency. The findings are not limited to sorghum-based biorefineries but extend to other grains and lignocellulosic feedstocks, supporting the development of future biorefineries designed for the co-utilization of natural feedstocks, such as wheat, sorghum, corn, rice, etc. This work thus offers a significant foundation for sustainable and efficient industrial bioprocessing innovations. Future research should focus on scaling up the process to pilot and industrial levels, optimizing the integration of enzyme production and biomass hydrolysis. In addition, genetic and metabolic engineering of fungal strains could further enhance enzyme yields and process robustness, accelerating the development of next-generation biorefineries.

CRediT authorship contribution statement

Pratul Dipta Somadder: Writing – review & editing, Writing – original draft, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Guangnan Chen:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Investigation. **Amin Mojiri:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis. **John Dearnaley:** Writing – review & editing, Visualization, Resources, Methodology. **Antoine Trzcinski:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: There is no conflict of interest If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Data availability

Data will be made available on request.

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