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Bioethanol production from cassava starch using co-culture of saccharolytic molds with *Saccharomyces cerevisiae* TISTR 5088

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ABSTRACT: Cassava serves as a cornerstone for sustainable bioethanol production in Thailand. The cassava root is highly valuable, comprising 50–70% starch on a dry weight basis. Starch can be converted into fermentable sugars through chemical or biological methods. This study focused on harnessing the enzymatic potential of saccharolytic molds, specifically *Aspergillus oryzae* TISTR 3086 or *Amylomyces rouxii* TISTR 3182, to facilitate saccharification of cassava starch, followed by fermentation using *Saccharomyces cerevisiae* TISTR 5088 for bioethanol production. Among the various configurations, the co-culture of *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 was the most effective, resulting in the highest ethanol yield. This finding was observed for both the separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes. Impressively, the SSF approach to ethanol productivity demonstrated superior results with an ethanol concentration of 25.4±0.3 g/l and an ethanol productivity of 0.53 g/l/h (yield of 1.40 g/g reducing sugars), surpassing the SHF approach (25.4±0.7 g/l, an ethanol productivity of 0.26 g/l/h, yield of 0.57 g/g reducing sugars) while also reducing the fermentation period. Further investigations into optimizing the conditions for ethanol production were carried out using a co-culture of *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 during SSF. This exploration revealed that employing 100 g/l cassava starch and initiating fermentation with a medium pH of 6.0 led to the highest ethanol concentration at 48 h. This process showed potential for ethanol production, harnessing the synergistic action of saccharolytic molds alongside yeast.

KEYWORDS: *Amylomyces rouxii*, bioethanol production, cassava, co-culture, *Saccharomyces cerevisiae*

INTRODUCTION

The surge in industrialization and population growth has precipitated a relentless increase in global energy demand. Currently, more than 80% of the world's energy production relies on fossil fuels. However, the rapid depletion of these resources, coupled with their detrimental effects on the environment and human health [1], underscores the urgent need for sustainable and renewable energy alternatives. In response, biofuels have emerged as a promising solution for meeting energy demands while minimizing environmental impacts [2]. Bioethanol, derived from renewable organic sources, is a viable alternative to conventional oil. It has gained prominence within the biofuel industry due to its ability to significantly reduce carbon dioxide emissions in the transport sector. Additionally, ethanol boasts a high energy content and is easily stored [3].

Cassava (*Manihot esculenta* Crantz), a staple crop with a starch content of approximately 63% [4], plays a significant role in this domain with Thailand alone producing approximately 32.73 million tons annually as of 2023 [5], highlighting its immense demand. In Thailand, cassava cultivation is categorized into 2 types: sweet and bitter varieties. Sweet cassavas contain minimal levels of hydrocyanic acid, whereas bitter cassavas contain high levels of this toxic compound, rendering them unsuitable for human consumption or direct animal feed [6]. The sample supply of cassava met the country's needs sufficiently. Moreover, cassava can be processed and transformed into value-added products such as methane (biogas) and ethanol [7, 8] with ethanol being extensively utilized worldwide, particularly in the transportation sector.

Ethanol production from starchy materials comprises 2 crucial stages: (i) starch liquefaction, facilitated by α-amylase, followed by enzymatic saccharification of the resulting low-molecular-weight products such as dextrin, yielding glucose and (ii) the fermentation of glucose into ethanol by specialized microorganisms [9]. Bioethanol production can be accomplished through 2 distinct processes: separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). SHF and SSF are the 2 dominant process configurations that employ enzymes for saccharification [10]. SHF allows hydrolysis and fermentation processes to be conducted separately. Enzymatic activity and microbial growth can be hindered by toxic and undesirable compounds, i.e., residual organic cyanide [11] present in the hydrolysates, which may originate from the lignocellulosic pretreatment process. Consequently, this inhibition can lead to a

reduction in ethanol productivity [12]. In contrast, SSF can simultaneously hydrolyze biomass and ferment it into bioethanol, thereby reducing hydrolysate inhibition. SSF requires a compromise between the optimum conditions for enzymatic hydrolysis and microbial fermentation [13].

In this context, this study aimed to evaluate these 2 processes for bioethanol fermentation using a co-culture approach. Here, saccharolytic molds, specifically *A. oryzae* TISTR 3086 and *Amy. rouxii* TISTR 3182, were paired with the fermentative capabilities of *S. cerevisiae* TISTR 5088. This strategic synergy aims to enhance the efficiency of bioethanol production by addressing key challenges associated with commercial enzymes. By integrating these distinct biological components, this study aspired to unlock a more sustainable and cost-effective path for bioethanol generation from cassava starch.

MATERIALS AND METHODS

Preparation of culture medium and inoculum

Amy. rouxii TISTR 3182 and *A. oryzae* TISTR 3086 were obtained from The Thailand Institute of Scientific and Technological Research. Strains TISTR 3182 and TISTR 3086 were cultivated on potato dextrose agar (PDA) and incubated at 30 °C for 7 days. Fungal spores were harvested by pouring 10 ml of sterile 0.1% Tween-80/water solution onto the agar surface containing the cells or spores and gently scraping with a surface-sterilized glass rod. The cell/spore suspensions were transferred to sterile 50-ml tubes and centrifuged at 2,000 rpm for 2 min, after which the supernatants were discarded. Fresh sterile 0.1% Tween-80/water was added. The concentration of fungal spores was determined using a hemacytometer (Marienfeld, Munich, Germany) and adjusted to approximately 10^8 spores/ml with sterile 0.1% Tween-80 [14]. *S. cerevisiae* TIST 5088 was cultured on YPD agar (20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract, and 15 g/l agar) and incubated at 30 °C for 72 h. One loopful was transferred to YPD broth and incubated at 30 °C for 24 h. The optical density (OD_{660}) was adjusted to 0.5 for use as the inoculum [15], resulting in a concentration of 10^6 CFU/ml.

Preparation of fermentation medium

Bitter cassava was sourced from a local market in Bangkok. Subsequently, it was cut into pieces and dried at 70 °C for 48 h. The dried cassava pieces were then milled into a fine powder and sifted through a 50-mesh sieve (Thermo Fisher Scientific, Dreieich, Germany) to obtain a powder size of 300 µm. A 60 g/l cassava starch solution was used as the medium for fermentation, and the initial pH was 5.00±0.02.

Determination of glucoamylase activity

The glucoamylase activity of the 2 molds was assessed. Two molds were streaked on potato dextrose agar (PDA) and incubated at 30 °C for 7 days. A 0.1% Tween 80 solution was used to disperse and suspend the spores. The spore suspension was counted by a hemacytometer and adjusted to 10^7 spores/ml. Ten milliliters of the spore suspension (10^7 spores/ml) was transferred to 90 ml of cassava starch broth and agitated at 150 rpm at 30 °C for 96 h in an incubator shaker (Labnet, New Jersey, USA). After incubation, the cell-free supernatants (CFSs) were centrifuged at 5,000 rpm at 4 °C for 15 min and subsequently subjected to glucoamylase activity assays following a method previously described by Ramadas et al [16]. The glucoamylase activity (GA) was determined on a glass test tube containing 0.1 ml of CFS, 0.4 ml of sodium acetate buffer (pH 4.0), and 0.5 ml of 1% (w/v) soluble starch and then incubated in a water bath at 60 °C for 10 min. Afterwards, 1.5 ml of the 3,5-dinitrosalicylic acid (DNS) solution was added, and the mixture was incubated at 100 °C for 5 min [17]. The absorbance was measured at 540 nm by a UV-visible spectrophotometer (Shimadzu, UV-1800, Japan). One unit of GA was defined as the amount of enzyme required to release 1 µmol of glucose/min under the assay conditions, and activity was expressed as U/ml.

Comparing bioethanol fermentation: co-cultures of saccharolytic molds and *S. cerevisiae* in the SHF and SSF processes

SHF process

Ethanol fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 150 ml of gelatinized cassava starch. The flasks were sterilized by autoclaving at 121 °C for 15 min. After sterilization, a 10% (v/v) inoculum of *Amy. rouxii* TISTR 3182 and *A. oryzae* TISTR 3086 spore suspensions were added. The culture was then incubated at 30 °C with agitation at 150 rpm for 24 h in an incubator shaker. Before adding yeast, the enzyme was inactivated by heating at 90 °C for 10 min. Following this initial incubation, a 10% (v/v) inoculum of *S. cerevisiae* TISTR 5088 was added, and the co-culture was further incubated under the same conditions for 120 h.

SSF process

Batch fermentation was performed using 150 ml of gelatinized cassava starch. In this process, a co-culture was prepared by simultaneously inoculation of a 10% (v/v) spore suspension of *Amy. rouxii* TISTR 3182, *A. oryzae* TISTR 3086, and 10% (v/v) *S. cerevisiae* TISTR 5088. The mixture was then incubated under the same conditions as those in the SHF process, and fermentation was continued for 120 h.

Optimal conditions for bioethanol fermentation

Effect of substrate concentration

Solutions with different concentrations (40, 60, 80, and 100 g/l) of cassava starch were prepared by mixing cassava starch with water and subsequently gelatinizing the mixture at 90 °C for 30 min. For liquefied starch, a co-culture containing 10% (v/v) *Amy. rouxii* TISTR 3182 and 10% (v/v) *S. cerevisiae* TISTR 5088 was prepared. The fermentation mixture was incubated at 30 °C with agitation at 150 rpm for 120 h in an incubator shaker.

Effect of pH

After examining the effect of substrate concentration, the next step involved adjusting the pH of the liquefied cassava starch to different levels (pH 4, 5, and 6). The pH-adjusted mixture was then subjected to fermentation with an appropriate substrate concentration and inoculated with a co-culture comprising 10% (v/v) *Amy. rouxii* TISTR 3182 and 10% (v/v) *S. cerevisiae* TISTR 5088. Fermentation was conducted at 30 °C with agitation at 150 rpm for 120 h in an incubator shaker to assess the effect of the pH.

Analytical method

At 24-h intervals, the fermentation broth from triplicate flasks ($n = 3$) was extracted, and its contents were analyzed for both reducing sugar and ethanol concentrations. The ethanol concentration in the fermented broth was determined using a gas chromatograph (Shimadzu, model GC-2014, Japan) equipped with a DB-1 column (Agilent, Santa Clara, USA). Helium served as the carrier gas at a flow rate of 244.2 ml/min, and flame ionization detection (FID) was maintained at 180 °C. The column temperature was 60 °C, and 10% N-propanol was used as the internal standard. The concentration of the reducing sugar was assessed using DNS method [17], and the reduced sugar concentration was determined based on a glucose standard curve. Additionally, the pH was measured using a pH meter (Mettler-Toledo, USA).

Statistical analysis

All experiments followed a completely randomized design (CRD) and were conducted in triplicate. The bioethanol yield (g/g) was calculated based on the experiment and expressed as g of bioethanol per total g of reducing sugar [18]. The results were subjected to analysis of variance (one-way ANOVA) using SPSS software (version 22.0). Significant differences among the means ($p < 0.05$) were evaluated using Duncan's new multiple range test (DMRT).

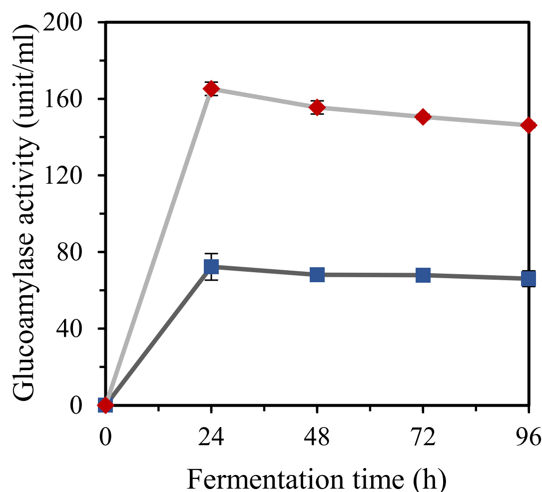

Fig. 1 Glucoamylase activities of saccharolytic molds; —■— *A. oryzae* TISTR 3086 and —◆— *Amy. rouxii* TISTR 3182.

RESULTS AND DISCUSSION

Determination of glucoamylase activity

In enzymatic catalysis, glucoamylase plays a central role in governing the hydrolysis of starch into glucose, a process of paramount importance in the food industry [19, 20]. This study focused on saccharolytic molds, specifically *A. oryzae* TISTR 3086 and *Amy. rouxii* TISTR 3182, and glucoamylase activity was assessed using the DNS method with 1% (w/v) starch as the substrate. After 24 h, *Amy. rouxii* TISTR 3182 exhibited robust extracellular glucoamylase activity (165.2±6.9 U/ml), surpassing that of *A. oryzae* TISTR 3086 (72.3±3.5 U/ml) (Fig. 1). These findings indicate a significant increase compared to previous studies, which also documented substantial glucoamylase activity in various strains, including *Amy. rouxii* PB03, SR02, and UD02 as well as *A. oryzae* UD01 and *Aspergillus niger* ST02 (ranging from 149.20 to 152.60 U/ml) [21]. These strains were isolated from a naturally fermented inoculum known as *Loog-pang-Khao-mak* in Thailand [21]. Similarly, Limtong et al [22], Daroonpunt et al [23], and Roongrojmongkhon et al [21] reported the frequent presence of *Amy. rouxii* and *Aspergillus* spp. within the ecological milieu of *Loog-pang-khao-mak*; they emphasized the heightened amylolytic activity exhibited by *Amy. rouxii* strains. Overall, this discussion highlights the importance of glucoamylase activity in starch hydrolysis. This study highlights the superior glucoamylase activity of *Amy. rouxii* TISTR 3182 compared to *A. oryzae* TISTR 3086, leading to higher levels of reducing sugars. This suggests the potential for more efficient starch hydrolysis into glucose, a crucial step in bioethanol production.

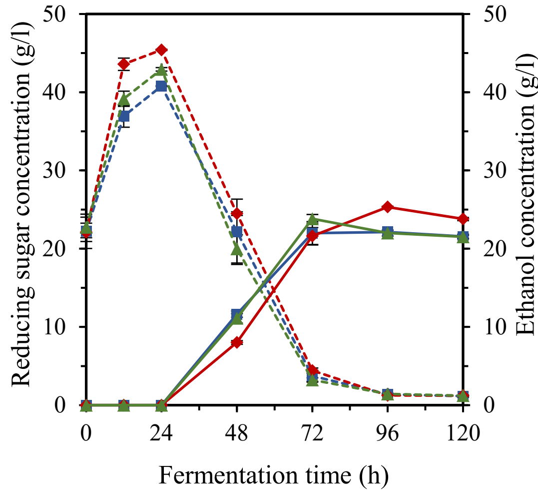

Fig. 2 Ethanol fermentation using a co-culture of saccharolytic molds with *S. cerevisiae* TISTR 5088 by the SHF process. Reducing sugar concentration (- - -) and ethanol concentration (——). —■— *A. oryzae* + *S. cerevisiae*, —◆— *Amy. rouxii* + *S. cerevisiae*, and —▲— *A. oryzae* + *Amy. rouxii* + *S. cerevisiae*.

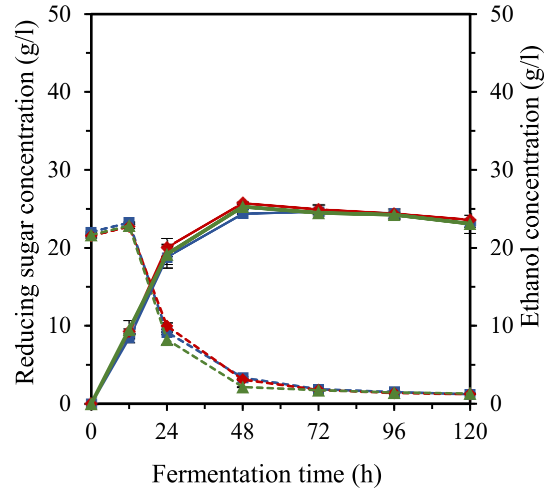

Fig. 3 Ethanol fermentation using a co-culture of saccharolytic molds with *S. cerevisiae* TISTR 5088 by SSF process. Reducing sugar concentration (- - -) and ethanol concentration (——). —■— *A. oryzae* + *S. cerevisiae*, —◆— *Amy. rouxii* + *S. cerevisiae*, and —▲— *A. oryzae* + *Amy. rouxii* + *S. cerevisiae*.

SHF bioethanol production: co-culture of saccharolytic molds with *S. cerevisiae* TISTR 5088

The experiment utilized a substrate composed of cassava starch at an initial concentration of 60 g/l. The fermentation process involved a co-culture approach, initially using 10% (v/v) *Amy. rouxii* TISTR 3182 or *A. oryzae* TISTR 3086 to initiate the hydrolysis of cassava starch. After 24 h, 10% (v/v) *S. cerevisiae* TISTR 5088 was added to the hydrolyzed cassava starch to convert sugars into ethanol. Fermentation was carried out at a temperature of 30 °C with agitation at 150 rpm for 120 h. The results are shown in Fig. 2.

The co-culture of *Amy. rouxii* TISTR 3182 and *S. cerevisiae* TISTR 5088 exhibited higher ethanol concentrations, reaching 25.3±0.1 g/l, an ethanol productivity (Qp) of 0.26 g/l/h and a yield of 0.57 g/g reducing sugars, than did the combination of *A. oryzae* TISTR 3086 with *S. cerevisiae* TISTR 5088, and the co-culture of *Amy. rouxii* TISTR 3182 and *A. oryzae* TISTR 3086 with *S. cerevisiae* TISTR 5088 improved ethanol concentrations by 22.2±0.0 g/l (Qp 0.23 g/l/h, yield 0.56 g/g reducing sugar) and 22.0±0.4 g/l (Qp 0.23 g/l/h, yield 0.50 g/g reducing sugar), respectively, within a 96-h timeframe. Kinetics of ethanol fermentation using a co-culture of saccharolytic molds with *S. cerevisiae* TISTR 5088 in SHF processes is shown in Table 1.

The reduced sugar levels initially increased for up to 24 h of fermentation, followed by a subsequent decrease. This trend could be attributed to the enzymatic hydrolysis of cassava starch by saccharolytic molds, which produced reducing sugars during the first 24 h. Notably, *Amy. rouxii* TISTR 3182 exhibited superior glucoamylase activity to that of *A. oryzae* TISTR 3086 (Fig. 2). Consequently, at 24 h, *Amy. rouxii* TISTR 3182 generated a greater quantity of reducing sugars (45.4±0.1 g/l) than did *A. oryzae* TISTR 3086 (40.8±0.1 g/l) and the co-culture of *Amy. rouxii* TISTR 3182 and *A. oryzae* TISTR 3086 with *S. cerevisiae* TISTR 5088 (42.9±0.2 g/l), as illustrated in Fig. 2. Consequently, *S. cerevisiae* TISTR 5088 predominantly utilizes the available reducing sugars for ethanol production. In terms of substrate utilization and ethanol production, *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 exhibited superior efficiency compared with other combinations involving *A. oryzae* TISTR 3086. A comparative study was conducted to analyze ethanol production by our strain *Amy. rouxii* TISTR 3182, *S. cerevisiae* TISTR 5088, and other yeasts, as documented in previous literature. For example, *Pichia kudriavzevii* strains CPY514-1, PBB511-1, TM512-2, and TG514-2 displayed ethanol yields of 0.13, 0.15, 0.15, and 0.13 g/g, respectively, from cassava starch hydrolysate containing 18% (w/v) reducing sugar at high temperature (45 °C) [24, 25].

SSF bioethanol production: co-culture of saccharolytic molds with *S. cerevisiae* TISTR 5088

Throughout the SSF process, the ethanol concentration steadily increased. At the 48-h mark, the co-culture of *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 exhibited the highest ethanol concentration, representing 25.7±0.1 g/l (Qp 0.54 g/l/h, yield 1.40 g/g reducing sugar). As shown in Table 1, the co-culture

of *A. oryzae* TISTR 3086 with *S. cerevisiae* TISTR 5088 exhibited an ethanol concentration of 24.4±0.1 g/l (Qp 0.51 g/l/h, yield 1.30 g/g reducing sugar), and the ethanol concentration for the co-culture of *Amy. rouxii* TISTR 3182 and *A. oryzae* TISTR 3086 with *S. cerevisiae* TISTR 5088 was 25.3±0.3 g/l (Qp 0.53 g/l/h, yield 1.29 g/g reducing sugar) (Fig. 3). The ethanol concentration remained relatively stable beyond the 48-h mark. The decrease in sugar consumption was correlated with ethanol concentration, as yeast utilizes reducing sugars as a carbon source, simultaneously contributing to mold growth. Co-culturing *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 resulted in higher ethanol concentrations than the other combinations. This indicates improved efficiency in converting sugars into ethanol, a key metric for bioethanol production.

In the early stages of the SSF process, a noticeable increase in the reduced sugar concentration was observed, suggesting that hydrolysis occurred faster than ethanol fermentation. By the culmination of the SSF process, the residual reducing sugar levels ranged from 1.21 to 1.32 g/l (Fig. 3), indicating nearly complete sugar consumption by the end of the fermentation period. Our study underscores the superior ethanol productivity achieved through the SSF process in comparison to the SHF process. The SSF process demonstrated a greater ethanol concentration and yield than did the SHF process. This suggests that SSF could be a more economically viable and energy-efficient approach for bioethanol production.

This finding is consistent with the observations of Phachanseesoulath et al [26], who reported that *S. cerevisiae* EC1118 could achieve ethanol concentrations and yields of 133.6 g/l and 0.51 g/g, respectively, when fermenting a high concentration of cassava (30%, w/v) using the SSF process. They also documented a similar trend characterized by higher initial ethanol productivity and reduced glycerol accumulation in the SSF process. The initial glucose concentration is one of several factors impacting ethanol production efficiency. Elevated glucose levels can induce stress in yeast cells, particularly when the cell density is initially low. This stress, in turn, can impede fermentation in processes involving high solid content. During the SSF process, it is essential to maintain an optimal glucose concentration to avoid inhibition, as yeast cells rapidly utilize hydrolyzed glucose [27–29].

Furthermore, this study revealed a greater ethanol concentration than a previous investigation. Specifically, in the SSF process, bioethanol production from cassava starch (6% v/v) utilizing *S. cerevisiae* TISTR 5088 and *Rhizopus* sp. #3Su yielded the highest reducing sugar content of 25.9% with peak ethanol production reaching 14.36 g/l [30]. Additionally, in our comparison between SSF and SHF, SSF demonstrated a higher ethanol concentration and yield. This observation is supported by Tomás-Pejó et al [12], who also compared SSF methodologies. In their research, they achieved the highest ethanol concentration of 23.7 g/l using the whole slurry (10% w/v) and *S. cerevisiae* F12 in an SSF process with an ethanol yield on consumed sugars of 0.43 g/g. Notably, ethanol production in the SSF process occurred significantly faster than that in the SHF process, suggesting lower enzyme inhibition by glucose. Additionally, due to the higher enzyme activity observed in SSF, we explored the possibility of using a smaller quantity of enzyme.

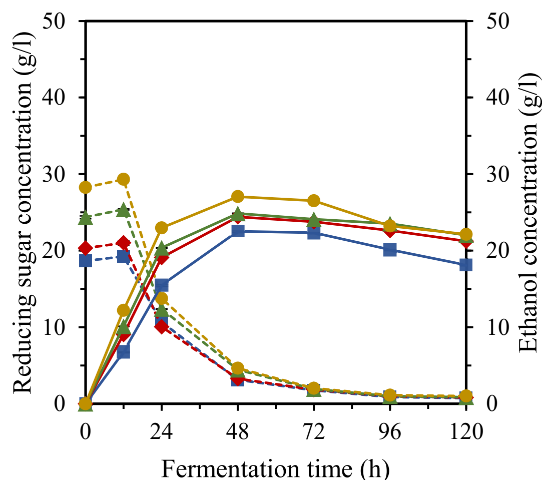

Fig. 4 Bioethanol fermentation of cassava starch at different initial starch concentrations. Reducing sugar concentration (- - -) and ethanol concentration (——). —■— 40 g/l starch, —◆— 60 g/l starch, —▲— 80 g/l starch, and —●— 100 g/l starch.

Optimization of ethanol fermentation conditions

Effect of the starch concentration on ethanol fermentation

The effect of the initial cassava starch concentration on ethanol fermentation was thoroughly investigated. Mixtures with different cassava starch concentrations (40, 60, 80, and 100 g/l) were prepared in water and subjected to gelatinization at 90 °C for 30 min. Subsequently, the mixtures were autoclaved at 121 °C for 15 min and inoculated simultaneously with 10% (v/v) *Amy. rouxii* TISTR 3182 and 10% (v/v) *S. cerevisiae* TISTR 5088 for ethanol fermentation. The results are shown in Fig. 4 and Table 2. The initial substrate concentration notably influenced the ethanol fermentation outcomes. Higher substrate concentrations were significantly more favorable, resulting in final ethanol concentrations of 27.1±0.03 g/l, 24.9±0.04 g/l, 24.4±0.04 g/l, and 22.5±0.11 g/l for starch concentrations of 100, 80, 60, and 40 g/l, respectively, at the 48-h mark (Fig. 4).

Mojović et al [31] reported that lower substrate

concentrations (11.5% w/v) were more conducive to ethanol fermentation, primarily to avoid substrate inhibition. Furthermore, the inhibition of ethanol production can stem from various factors, including elevated sugars, ethanol, and salts, and the presence of raw materials or substances generated during the pretreatment/hydrolysis process [32]. As shown in Fig. 4, the maximum ethanol concentration (27.1±0.03 g/l) and ethanol productivity (0.56 g/l/h) were achieved at the highest initial substrate concentration within the mixture (100 g/l). However, considering the practical implications of using high-viscosity starch for ethanol production is crucial. High-viscosity starch can hinder ethanol production by affecting various stages, including saccharification and fermentation. It resists enzymatic hydrolysis, which leads to lower sugar yields and reduced efficiency. Mixing and agitation have become more demanding, potentially increasing the cost and fermentation time. A thicker fermentation broth can also hinder downstream processing such as separation and distillation, affecting ethanol recovery rates and energy consumption [33]. Although high-viscosity starch may offer cost advantages, its practicality in ethanol production is limited, prompting the need to explore alternative starch sources or optimization methods. Moreover, high starch concentrations hinder the gelatinization process of starch [34]. The elevated glucose concentration in the fermentation media could intoxicate the yeast and decrease the fermentation rate. Additionally, high sugar concentrations lead to increased viscosity in the fermentation broth, which inhibits yeast growth, reduces sugar utilization, and diminishes the capacity for bioethanol production. Furthermore, yeast may face various environmental stresses, resulting in reduced viability, growth, and fermentation rates, thereby prolonging fermentation time [35]. Glucose is reduced to bioethanol, but there is also another reaction that produces pyruvic acid as a secondary product [36].

Effect of initial pH on ethanol fermentation

The initial pH is pivotal for influencing amylolytic enzyme activity and ethanol production. To investigate the impact of pH on ethanol fermentation, batch experiments were conducted at various pH values ranging from 4.0 to 6.0. Co-cultures of *Amy. rouxii* TISTR 3182 and *S. cerevisiae* TISTR 5088 were employed with an initial substrate concentration of 100 g/l and agitation set at 150 rpm over a 120-h fermentation period.

As illustrated in Fig. 5, the highest ethanol concentration (29.36±0.03 g/l), ethanol productivity (0.61 g/l/h), and ethanol yield (1.31 g/g substrate) were potentially achieved at pH 6.0. Similar findings were reported by Duhan et al [37], who reported a maximum ethanol yield of 7.70% in *S. cerevisiae* MTCC-170 cultured at pH 6.0. Mohanty et al [38] indicated that a pH of 6.0 is optimal for bioethanol

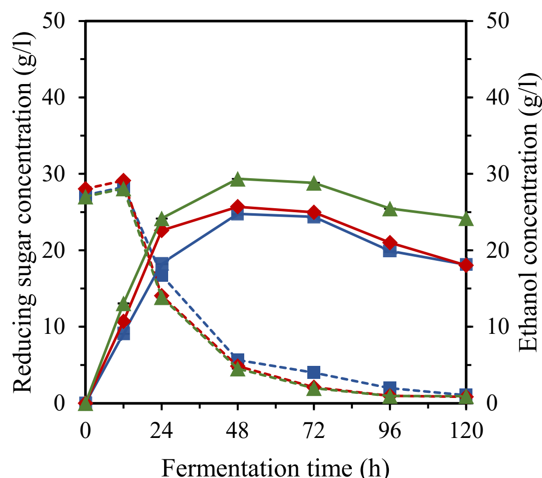

Fig. 5 Bioethanol fermentation of cassava starch at different initial pH values. Reducing sugar concentrations (- - -) and ethanol concentrations (——). —■— pH 4, —◆— pH 5, and —▲— pH 6.

production from Mahula flowers (*Madhuca latifolia* L.) via solid-state fermentation. Notably, yeast cells cannot thrive in acidic conditions, hindering their ability to consume fermentable sugars and produce ethanol. Yeast cells prefer mildly acidic to slightly alkaline environments for optimal growth and fermentation activity [39].

Under highly acidic or alkaline conditions, yeast cell growth may be impeded. This result was further supported by Tsuji et al [40], who reported that the optimum pH for bioethanol production is 4–10.5.

The study identified optimal conditions for ethanol fermentation, including higher initial substrate concentrations of cassava starch and maintaining an initial pH of 6.0. These findings contribute to improving the efficiency and productivity of bioethanol production processes and underscore the significance of process optimization in enhancing ethanol productivity. By optimizing conditions such as substrate concentration and pH, researchers can achieve higher ethanol yields, thus improving the overall efficiency of bioethanol production.

CONCLUSION

These findings revealed that *Amy. rouxii* TISTR 3182 exhibited superior glucoamylase activity compared to that of *A. oryzae* TISTR 3086, resulting in higher levels of reducing sugars and significantly contributing to increased ethanol production. In the SSF process, the co-culture of *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 demonstrated the highest ethanol concentration (25.4±0.3 g/l), highlighting the efficacy of this combination in terms of ethanol productivity. The SSF process outperformed the SHF process primarily because of its economic viability and reduced energy con-

Table 1 Kinetics of ethanol fermentation using a co-culture of saccharolytic molds with *S. cerevisiae* TISTR 5088 compared between the SHF and SSF processes.

Fermentation kinetics	SHF process (at 96 h)			SSF process (at 48 h)		
	A. oryzae + S. cerevisiae	*Amy. rouxii + S. cerevisiae*	*A. oryzae + Amy. rouxii + S. cerevisiae*	*A. oryzae + S. cerevisiae*	*Amy. rouxii + S. cerevisiae*	*A. oryzae + Amy. rouxii + S. cerevisiae*
C (g/l)	22.15 ± 0.01^{b}	25.33 ± 0.11^{a}	22.00 ± 0.37^{b}	24.35 ± 0.07^{c}	25.73 ± 0.14^{a}	25.27 ± 0.34^{b}
Qp (g/l/h)	0.24 ± 0.01^{b}	0.26 ± 0.01^{a}	0.23 ± 0.01^{b}	0.51 ± 0.02^{b}	0.54 ± 0.01^{a}	0.52 ± 0.01^{ab}
Yp/s (g/g)	0.56 ± 0.01^{a}	0.57 ± 0.01^{a}	0.53 ± 0.01^{b}	1.30 ± 0.02^{b}	1.40 ± 0.02^{a}	1.29 ± 0.01^{b}
Ty (g substrate)	20.85 ± 0.05^{c}	23.21 ± 0.02^{a}	21.94 ± 0.12^{b}	11.27 ± 0.05^{a}	11.04 ± 0.06^{b}	11.07 ± 0.03^{b}

Abbreviations: C, ethanol concentration (g/l); Qp, ethanol productivity (g/l/h); Yp/s, ethanol yield (g/g); and Ty, ethanol theoretical yield (g substrate). The mean values given in rows with different letter index are significantly different ($p < 0.05$).

Table 2 Kinetics of ethanol fermentation using a co-culture of *Amy. rouxii* TISTR 3182 with *S. cerevisiae* TISTR 5088 at different initial starch concentrations and pH values by the SSF process at 48 h.

Fermentation kinetics	Starch concentration (g/l)				pH		
	40 g/l starch	60 g/l starch	80 g/l starch	100 g/l starch	pH 4	pH 5	pH 6
C (g/l)	22.53 ± 0.11^{c}	24.42 ± 0.04^{b}	24.86 ± 0.04^{b}	27.06 ± 0.03^{a}	24.78 ± 0.06^{c}	25.70 ± 0.05^{b}	29.36 ± 0.03^{a}
Qp (g/l/h)	0.46 ± 0.02^{c}	0.51 ± 0.02^{b}	0.53 ± 0.02^{b}	0.57 ± 0.01^{a}	0.52 ± 0.01^{c}	0.55 ± 0.01^{b}	0.62 ± 0.01^{a}
Yp/s (g/g)	1.45 ± 0.01^{a}	1.42 ± 0.02^{a}	1.25 ± 0.02^{b}	1.16 ± 0.02^{c}	1.13 ± 0.03^{b}	1.14 ± 0.07^{b}	1.32 ± 0.03^{a}
Ty (g substrate)	9.53 ± 0.02^{d}	10.42 ± 0.02^{c}	12.47 ± 0.29^{b}	14.43 ± 0.14^{a}	13.89 ± 0.03^{b}	14.35 ± 0.11^{a}	13.81 ± 0.08^{b}

Abbreviations: C, ethanol concentration (g/l); Qp, ethanol productivity (g/l/h); Yp/s, ethanol yield (g/g); and Ty, ethanol theoretical yield (g substrate). The mean values given in rows with different letter index are significantly different ($p < 0.05$).

sumption. Optimization experiments further revealed that higher initial substrate concentrations of cassava starch favored ethanol fermentation with 100 g/l yielding the maximum ethanol concentration, maintaining an initial pH of 6.0, and proving optimal for ethanol production in the co-culture system. In summary, this study underscores the significance of glucoamylase activity in saccharolytic molds, particularly *Amy. rouxii* TISTR 3182, in achieving efficient bioethanol production when co-cultured with *S. cerevisiae* TISTR 5088. Our study emphasizes the crucial role of process optimization in enhancing ethanol productivity.

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