Potential of R5I3 Isolate for Bioethanol Production on YPG Medium with Glucose and Fermentation Time Variations

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Abstract. Bioethanol production in Indonesia remains low despite the country's rich biomass potential, partly due to the lack of native ethanol-producing microbes. This study evaluated the potential of R5I3 isolate to produce ethanol using Yeast Peptone Glucose (YPG) medium with glucose concentrations ranging from 8–20% (w/v) and fermentation durations of 1–4 days. The results showed that R5I3 was capable of surviving and producing ethanol across all treatments. The highest ethanol yield was recorded in the 18% w/v glucose treatment with a fermentation period of 4 days, producing $9.75 \pm 0.35\%$ (v/v) ethanol, with a final pH of 3.47 ± 0.55, total dissolved solids of 6.30 ± 0.14°Brix, and reducing sugar of 0.688 ± 0.008 mg/mL. These findings indicate that R5I3 isolate has strong potential as a microbial agent for bioethanol production under varying glucose concentrations and fermentation times.

1 Introduction

The production of bioethanol in Indonesia remains comparatively low, particularly when viewed in the context of the country's abundant resources, which include a substantial amount of biomass waste from the agricultural and plantation sectors. At present, the annual bioethanol production capacity in Indonesia is approximately 40,000 KL [1]. Nevertheless, this production remains significantly below the government's target. A production goal of 1.2 million KL has been established for 2030, with expectations of reducing fuel import dependency by 60%, mainly gasoline, which recorded 35.8 million KL in 2022 [1].

The advancement of bioethanol in Indonesia encounters several obstacles. A major constraint lies in the limited supply of fermentation substrates, particularly molasses, which has traditionally served as the primary carbon source. As a by-product of the sugar industry, molasses also holds considerable economic value for other sectors such as the food industry and export markets, resulting in strong competition for its utilization [2]. Furthermore, the higher production cost of bioethanol relative to gasoline, the lack of government financial

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incentives, and insufficient distribution infrastructure further hinder industrial development. Another critical limitation is the shortage of locally available microbial isolates with high efficiency in biomass-to-ethanol conversion. Addressing these challenges is essential to advancing fermentation technologies that meet the demands of both economic feasibility and environmental sustainability.

For many years, bioethanol production has mainly relied on the use of microorganisms such as *Saccharomyces cerevisiae* [3,4]. This species is widely recognized for its ability to efficiently ferment simple sugars, particularly glucose and fructose. Other microorganisms are also employed in various production systems, including *Zymomonas mobilis*, which is valued for its efficiency through the Entner–Doudoroff pathway and its tolerance to relatively high ethanol levels. Even so, the use of these microorganisms is not without drawbacks, as they remain sensitive to changes in environmental conditions and often perform poorly when processing complex substrates.

Therefore, investigating and applying local microbial isolates with enhanced fermentative abilities is highly important. Such isolates may possess greater adaptability to tropical environments and the substrates commonly available in these regions. Previous studies have indicated that the R5I3 isolate represents a promising option for ethanol production. Gunam et al. [5] reported that the R5I3 isolate demonstrated the capacity to produce ethanol at a concentration of 8.63 ± 0.04% (v/v) in a yeast extract peptone glucose (YPG) medium containing 15% glucose, with a fermentation time of 4 days. Furthermore, research conducted by Sujana et al. [6] demonstrated that the same isolate was capable of producing ethanol at a rate of 5.39 ± 0.21% (v/v) from coconut sap, with no observed change in fermentation duration. Based on previous observations, it is reasonable to hypothesize that the R5I3 isolate has considerable potential for ethanol fermentation. Nonetheless, its performance under different glucose concentrations and fermentation durations remains unclear, thereby necessitating further investigation.

In consideration of the previously mentioned background, the objective of this study is to examine the effect of glucose concentration and fermentation time on the ability of the R5I3 isolate to produce ethanol. The glucose concentrations employed in this study range from 8 to 20% (w/v), while the fermentation durations examined span 1 to 4 days. It is hypothesized that this study will provide significant information regarding the optimal fermentation conditions for the R5I3 isolate. Furthermore, it is predicted that the study will enrich the collection of potential microorganisms that can be utilized for bioethanol production based on local resources. Formatting the title, authors and affiliations

2 Materials and Methods

2.1 Materials and Apparatus

This research utilized the R5I3 isolate, a locally obtained strain capable of converting glucose into ethanol. The isolate was previously characterized and preserved at the Bioindustry Laboratory, Faculty of Agricultural Technology, Udayana University. The other materials employed were peptone (Merck), yeast extract (Merck), sodium chloride (Merck), glucose (Lihua Starch), acetate buffer at pH 5.5, DNS reagent (Sigma-Aldrich), buffer solution at pH 7, distilled water, and several additional analytical reagents.

The equipment used in this study include Erlenmeyer (Iwaki), glass beaker (Herma), measuring cup (Pyrex), shaker rotator (health H-MSR), laminar air flow (Vienna Airflow), autoclave (Hirayama), magnetic stirrer (IKA), UV-Vis spectrophotometer (Thermo scientific), vortex (Maxi Max II), waterbath shaker (IKA), hand refractometer (Atago), pH-meter (Senz pH), centrifuge (Herolab), analytical balance, micro pipette, and other glassware.

2.2 Experiment design

This study employed an experimental approach using a randomized block design (RBD) with two treatment factors. The first factor was glucose concentration (G), while the second was fermentation time (T). Type glucose concentration (G) consisting of six levels, namely: G8: 8 %w/v; G10: 10 %w/v; G13:13 % w/v; G15: 15 %v/v; G18 %v/v; and G20: 20 %v/v. The second factor was the time of fermentation (T) which consists of four levels, namely: T1: 1 day; T2: 2 days; T3: 3 days; and T4: 4 days. Based on the description above, 24 treatment combinations were obtained. Each treatment was grouped into 2 groups based on the time of implementation, resulting in 48 experimental units.

2.3 Microorganism

R5I3 isolate was maintained on yeast peptone glucose (YPG) medium [5,6]. YPG media was made with the composition of yeast extract 0.45%, peptone 0.75%, and glucose 5% into distilled water as much as 100 mL. Culture rejuvenation, by taking 1 mL of R5I3 isolate from the stock culture, then transferred to 9 mL of media, after which it was incubated at 35°C for 24 h. The next stage was cell multiplication on new media with a larger volume of 50 mL, incubated at 35°C and shaker at 100 rpm for 48 h. After incubation, the cell suspension was aseptically harvested by centrifugation (3500 rpm, 5 min), the supernatant (YPG medium) was discarded, and the cells were washed twice in 10 mL of 0.85% (w/v) NaCl to minimize nutrient transfer from the seed culture to the fermentation medium [5,6].

2.4 Fermentation

During the fermentation stage, the substrate utilized is YPG medium, with modifications to its glucose concentration. A total of 200 mL of sterile YPG medium (pH adjusted to pH 5.5) is added to the fermenter bottle, along with 1% v/v of R5I3 starter culture [5]. Subsequently, the fermentation process is conducted for a duration of 1–4 days at a temperature of 35°C. Following the conclusion of the fermentation process, the distillation stage is initiated at a temperature range of 75–80°C [6].

2.5 Observed variable

The variables observed in this study were pH [7], total reducing sugar [8], total dissolved solids [9], and bioethanol content analysis [5,6]

2.6 Data analysis

The data obtained from the fermentation experiments were processed and analysed using Microsoft Excel. The analysis involved calculating the average values of each treatment and comparing the results based on different glucose concentrations and fermentation durations. The processed data were then presented in the form of bar charts to visually illustrate the differences in bioethanol production across treatments.

3 Results and discussion

3.1 pH

The initial pH value for all treatments was the same, namely pH 5.5, because all treatments were first adjusted to pH. The results of the pH changes in each treatment can be seen in Figure 1.

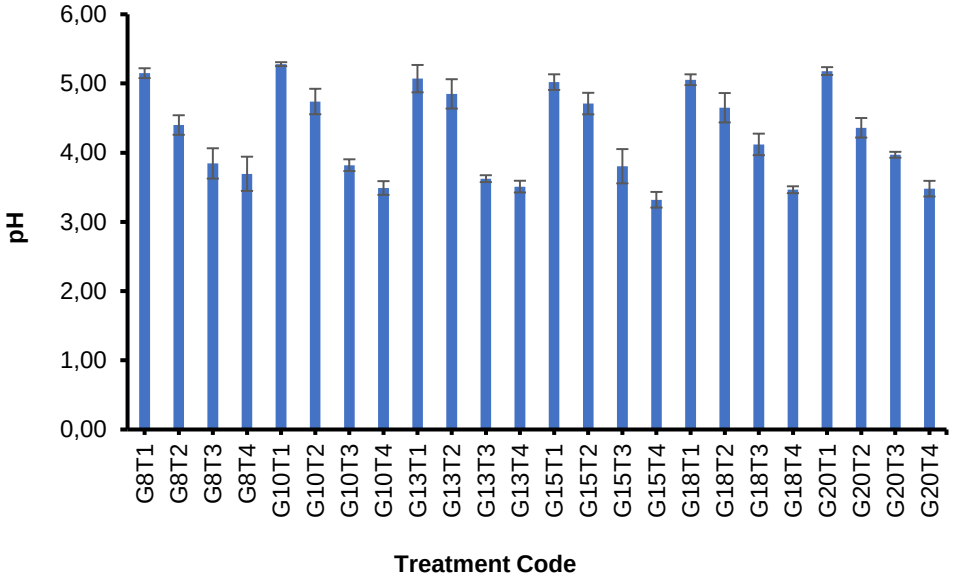

Fig. 1. Final pH value at each treatment

As illustrated in Figure 1, the glucose treatments exhibited a decline in response to an increase in the fermentation period. However, a notable absence of statistical significance was observed in the comparison of the pH values produced and the glucose concentrations utilized. In the study by Gunam et al., [5], isolate R5I3, after being fermented with a glucose concentration of 15% w/v and an initial pH of 5.5, produced a final pH ranging from 3.5 to 4 on the fourth day of fermentation.

The decrease in pH value as fermentation time increases indicates increased metabolic activity of microorganisms, which produce acidic compounds such as organic acids as fermentation by-products [5,6,10]. This phenomenon leads to an increase in the acidity of the environment, resulting in a tendency for pH values to decrease during extended fermentation periods. While glucose does not directly lower pH, higher glucose concentrations can increase microbial metabolic rates, which ultimately accelerates the formation of acidic compounds and indirectly contributes to pH reduction during the fermentation process [6]. Furthermore, the similarity in pH values among glucose treatments is attributable to the pre-fermentation adjustment of pH to its optimal level. This suggests that microorganisms are capable of thriving without the need to adjust the pH to optimal levels for their growth.

3.2 Total dissolved solid

The initial TDS values for all treatments varied, because this study used different glucose concentrations. Thus, the initial total dissolved solids values had different TDS values. The final TDS change values for each treatment can be seen in Figure 2.

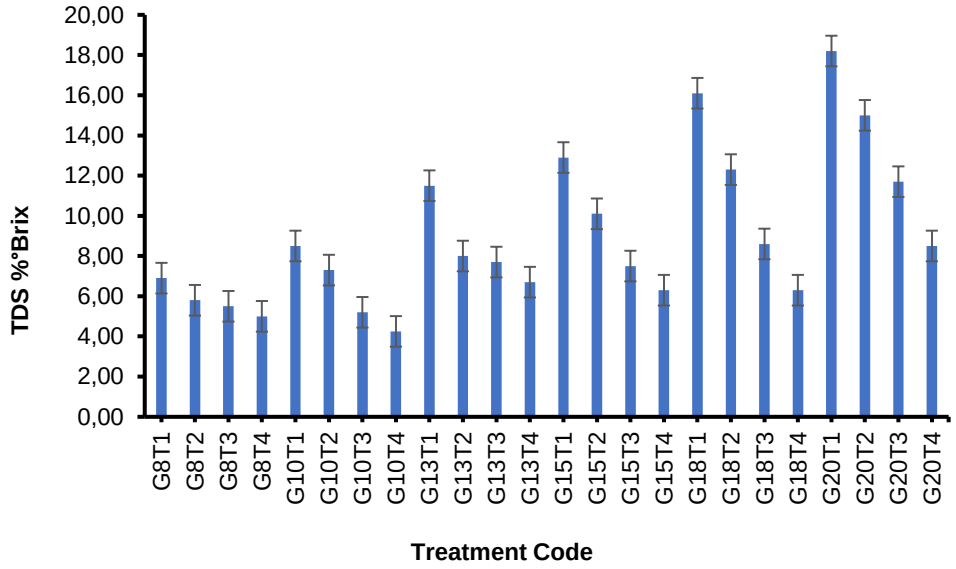

Fig. 2. Total dissolved solids value at each treatment

The results of this study indicate that fermentation time plays an important role in determining the final total dissolved solids (TDS) content. A longer fermentation period generally led to a decrease in TDS values [11]. This trend reflects microbial activity, particularly the consumption of glucose as the main substrate during fermentation [6]. As the process continues, more substrate is converted into ethanol and other metabolites, leaving fewer dissolved compounds in the medium.

Besides fermentation time, the initial glucose concentration was also found to be a determining factor for the final TDS value. As shown in Figure 2, the reduction in TDS at low glucose levels (e.g., G8 – 8% w/v) was not statistically significant, most likely due to the limited substrate availability that restricted microbial metabolism [12]. In contrast, higher glucose levels, such as 18% (w/v), supported a more pronounced decline in TDS, suggesting that abundant substrate allowed continuous and intensive microbial activity. Interestingly, in the G20 treatment (20% w/v glucose) after four days of fermentation (T4), the TDS value remained relatively higher than in other treatments. This suggests that although glucose was abundant at the start, the microorganisms required more time to fully utilize it. Fermentation under high substrate concentrations tends to proceed more slowly, partly due to osmotic pressure effects that can limit microbial growth and enzymatic performance [5]. This can result in the development of osmotic pressure, which has the potential to impede microbial proliferation and enzymatic activity. Therefore, the efficiency of fermentation is influenced by the balance between substrate availability and the microorganisms' ability to consume it optimally within a specific timeframe.

3.3 Total reducing sugar

The total reducing sugar value produced is the final reducing sugar, where the sample is tested before distillation. The final total reducing sugar value can be seen in Figure 3.

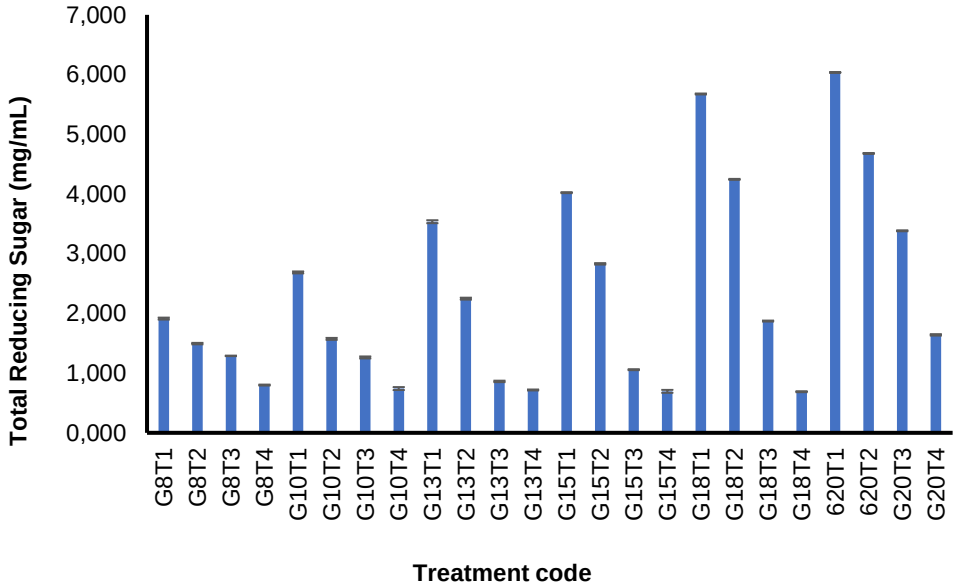

Figure 3. Final value of total reducing sugars produced at each treatment

As illustrated in Figure 3, an increase in fermentation duration is associated with a decrease in reducing sugar content. This phenomenon is analogous to the pH and TDS values, wherein an extended fermentation duration results in diminished values. It has been demonstrated that, in addition to the duration of fermentation, the initial glucose content also exerts a significant influence on the reduction in reducing sugar value. It has been demonstrated that an elevated initial glucose content results in a more rapid and substantial reduction in reducing sugar value.

As demonstrated in Figure 3, there is a clear trend of decreasing total reducing sugar with increasing fermentation time for each glucose concentration treatment. In the G8 group (8% w/v), there is a gradual decrease from day-1 (G8T1) until day-4 (G8T4), indicating consistent but relatively limited fermentative activity. A similar trend is observed in G10 and G13, with minor fluctuations, but the decrease continues progressively. In G15 and G18, a substantial decline in reducing sugars was observed from day 2 to day 3, followed by a pronounced decrease until T4.

However, the G20 treatment (20% w/v) exhibited a divergent pattern. The TRS value in G20T1 was found to be elevated, reaching nearly 6.5 mg/mL. Despite a gradual decline, the value in G20T4 remained comparatively high, at approximately 2.0 mg/mL, when compared to other treatment groups. This finding suggests that fermentation under high glucose conditions may not occur optimally, potentially due to high osmotic pressure impeding microbial metabolic activity or the Crabtree effect [13]. Excess glucose has been shown to induce a shift in cellular metabolism, favouring fermentation over respiration. However, this transition is characterized by low efficiency. Excessive glucose concentrations result in the accumulation of residual reducing sugars due to limitations in the cells' capacity to transport and metabolize glucose [6].

The findings of this study demonstrate that glucose levels have a significant impact on the outcomes observed. In instances where glucose levels are either excessively low or elevated, the reduction value is prone to being diminished. Consequently, while elevated initial glucose levels can augment the capacity for

bioethanol production, optimal fermentation efficiency is attained when glucose concentrations are within the optimal range (approximately 13–18% w/v) and fermentation time is sufficient (≥ 3–4 days).

3.4 Total ethanol

The pH, TDS, and total reducing sugar values are results that can support the total ethanol produced in each treatment. The total ethanol values obtained from this study can be seen in Figure 4.

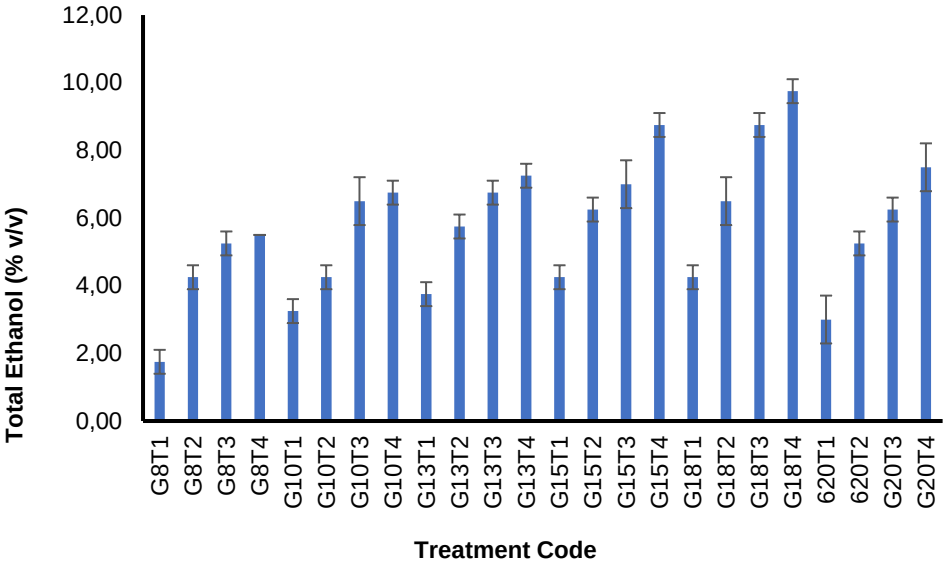

Fig. 4. Total ethanol value at each treatment

As illustrated in Figure 4, the outcomes of the study deviated from those obtained from the pH, TDS, and reducing sugar tests. In these experiments, it was observed that an increase in fermentation duration resulted in a decrease in the values obtained. However, this is distinct from the total ethanol produced, where an extended fermentation period results in a higher total ethanol yield. This phenomenon can be attributed to the increasing conversion of substrates into ethanol and the concurrent production of organic acids during prolonged fermentation periods [5,6,10]. A decrease in TDS or reduction in sugar levels has been demonstrated to increase ethanol yield. In the event of such a decrease, the fermentation process can proceed smoothly [5,6].

Furthermore, the glucose concentration has been demonstrated to influence the rate of ethanol production. In circumstances where glucose levels are sufficiently high, microbial enzymes are able to convert glucose into ethanol [6]. As demonstrated in Figure 4, in the event that the initial glucose concentration is minimal or below 10%, the production of ethanol will be negligible. However, if the glucose concentration is sufficient, ranging from 10–18%, it will produce good or optimal ethanol. Conversely, at glucose concentrations exceeding 18%, the ethanol yield exhibits a decline in comparison to the utilization of 18%.

The outcomes of this study demonstrate that the R5I3 isolate exhibits optimal glucose conversion efficiency at concentrations ranging from 15–18%, with a fermentation duration of 4 days. It is noteworthy that alternative treatments also yielded ethanol, albeit to a maximum of 15–18%, which is less than the aforementioned treatment. Furthermore,

evidence has emerged that the R5I3 isolate possesses the capacity to withstand both low and high glucose concentrations.

In the study by Sujana et al., [6], it was found that isolates IS258, R5I3, and *S. cerevisiae* ATCC 9763 were capable of producing ethanol at concentrations of 6.18 ± 0.45%, 5.39 ± 0.21%, and 4.65 ± 0.83%, respectively, using coconut sap as the substrate in the fermentation process. Previous studies have demonstrated varying ethanol production capacities among different microorganisms. For instance, Megawati et al., [14] obtained 4.80 g/L ethanol with *Saccharomyces cerevisiae*, whereas Manyuchi et al., [15] reported more than 40 mL/L with *Bacillus flexus* after 10 days of fermentation. In comparison with previous studies, the current results show that isolate R5I3 exhibits a notable capacity for ethanol production, emphasizing its promise for future bioethanol applications.

4 Conclusions

In this research, the ethanol-producing capacity of isolate R5I3 was evaluated. The findings indicated that the isolate was capable of surviving and synthesizing ethanol under different glucose concentrations and fermentation periods. The maximum ethanol yield was recorded in the G18T4 treatment, corresponding to 18% (w/v) glucose concentration and 4 days of fermentation, resulting in 9.75 ± 0.35% (v/v) ethanol. At this condition, the final pH was 3.47 ± 0.55, the total dissolved solids reached 6.30 ± 0.14% Brix, and the residual reducing sugar was 0.688 ± 0.008 mg/mL. These results suggest that isolate R5I3 has potential as an effective biocatalyst for glucose-to-ethanol conversion across a range of glucose levels.

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