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RESEARCH ARTICLE
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Potential Yeast from *Lau* as an Ethanol Fermentation Starter from the Arrack Industry in Bali, Indonesia

I M. Mahaputra Wijaya [1,2,*], Ida Bagus Wayan Gunam [1,2], Nyoman Semadi Antara [1,2], Ida Ayu Elma Wulandari Putri [1,3], I Gede Arya Sujana [1,2], Ni Putu Suwariani [1,2] and Gusti Ayu Kadek Diah Puspawati [2]

[1]Laboratory of Bioindustry, Faculty of Agricultural Technology, Udayana University, Denpasar Bali, Indonesia, 80234
[2]Faculty of Agricultural Technology, GA Building, Udayana University, Jimbaran Campus, Bali, Indonesia, 80361
[3]Indonesia Open University Unit of Distance Learning Program, Denpasar-Indonesia, 80361
*Corresponding author: mahaputrawijaya@unud.ac.id

ABSTRACT

Arak Bali is a well-known spirit in Indonesia, which is made from coconut sap using a traditional fermentation method. However, the microbe responsible for converting coconut sap sugar into ethanol in *arak* has yet to be clearly identified. This research aimed to elucidate the potential of the highest-performing microbe found in the traditional starter called *lau* in the arak (*arrack*) industry in Tri Eka Buana-Karangasem, Bali, named IS258. The IS258 isolate, previously assumed to be yeast, was found capable of producing 8.9% (*v/v*) ethanol in unoptimized rich PYG (peptone, yeast extract, and glucose) fermentation media. In this study, Response Surface Methodology (RSM) was utilized to test and optimize the ethanol yield using various fermentation media compositions. This work aimed to maximize the yield of the yeast IS258, which resulted in 11.2% (*v/v*) ethanol/liter of media with 20% glucose, and 84% stoichiometric glucose conversion rate. The genetic analysis conducted in this work revealed that IS258 belongs to the *Nakaseomyces glabrata*, known as *Candida glabrata*, which is widely recognized as an opportunistic pathogen, despite being found on many fermented foods in Southeast Asia. The discoveries found in this work suggest IS258's potential use as a bioethanol fermentation starter for the biofuel industry.

Keywords: *Arak Bali*, Bioethanol, Fermentation, Starter, DNA identification

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INTRODUCTION

Arak Bali (Balinese Arrack) is well known traditional spirit in Indonesia, where mostly produced by home industries in Karangasem Regency in Bali Province. *Arak Bali* is made from harvested coconut sap which traditionally fermented into ethanol, and followed by distillation. The fermentation process of sugar in coconut sap into ethanol is started by addition of *lau*, a traditional fermentation starter specifically used for making *Tuak Bali*, a Balinese drink made from fermented coconut sap with low alcohol content, or later distilled to produce spirit-type drink called *Arak Bali*. *Lau* was usually made of dried materials: shredded and tied coconut fiber (*Cocos nucifera*), bark of the local *bayur* tree (*Pterospermum javanicum*), or betel vine branch (*Piper betle*) (Simbolon et al., 2018).

Lau as fermentation starter was added into the sap container before coconut sap harvesting begun, thus the fermentation begun right after the sap was dripping into the container. The next day, all the sap in containers were collected from the coconut tree and transferred into larger container to continue the fermentation process for 2–4 days in tropical room temperature without any temperature conditioning. The fermented sap, called *Tuak Bali* was then distilled using single stage traditional distiller into ethanol spirit known as *Arak Bali*, with varying ethanol content of 15–30% (Hakim et al., 2020). The ethanol in *Tuak Bali* could yielded varying around 4–5% (*v/v*) from local coconut sap, which measured to contains around 14% (*w/v*) sugar (Prameshwari et al., 2024). The traditional *Arak Bali* distillery in Karangasem produced *Arak Bali* without sugar nor other nutrition as addition upon fermentation and conducted in non-aseptic condition.

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With the *lau* as the only addition into the sap to start the ethanol fermentation, it could be speculated that the microbes that functions as fermentation starter was exist in *lau* before the addition into the sap, despite accidental airborne addition of microbes into the sap upon harvesting and transport cannot be discounted.

Lau was made simply by drying the materials for few days under the sun in open air during dry season, without any other further treatment. The relatively high ethanol yield upon *Arak Bali* production from coconut sap gave a strong indication that potentially ethanol-producing microbes exist in *lau*. Since nothing was added into *lau* upon drying, possibly the microbes or spores were fell and adsorbed onto the *lau*. The physical condition of *lau* which consist of shredded coconut fiber or thin *Bayur* tree bark, with its high surface area might facilitates the adsorption of microbes or spore into its surfaces. However, given the non-aseptic nature of the *lau* drying process, the adsorption of both beneficial or disadvantageous microbes and spore cannot be controlled. Thus, various microbes might exist in fermented sap as contaminant, as result of the non-aseptic *lau* used as fermentation starter, where most microbes might not beneficial for sugar fermentation into ethanol, rendering the commonly conducted batch-process based traditional fermentation process (Wijaya et al., 2023), is not highly efficient.

Previously, several ethanol producing bacteria which presumed to be falling from the air were collected from the air from ethanol distillery hamlet in Karangasem Regency Bali using PDA agar plates in petri dishes (Bagaskara et al., 2020; Hakim et al., 2020). However due to the nature of the *Bayur* tree bark and shredded coconut fiber contain anti-bacterial agent, the most responsible microbe is likely to be yeast, instead of bacteria (Simatupang et al., 2019). This was confirmed by usage of anti-bacterial agent to selectively inhibit the bacterial growth, several potential yeasts were isolated from ethanol distillery hamlet in Karangasem Regency, where the highest performing yeast isolate named IS258. The IS258 yeast was isolated from already used *lau* made from coconut fiber in already fully fermented coconut sap. This isolate able to produce the highest ethanol yield among other candidate as much as 8.9% (*v/v*) using unoptimized PYG fermentation media (Simbolon et al., 2018). Further characterization in fermentation media pH and temperature optimization revealed higher ethanol yield as much as 10% (*v/v*) (Prameshwari et al., 2024).

The potential of IS258 which was discovered from traditional *Arak Bali* industry, where non-aseptic traditional sap harvest method was usually used, with low fermentation efficiency due to possible high contaminations in both harvest and fermentation, which conducted in tropical temperature, yet still yielded quite high ethanol results is interesting to be elucidated. The existence of previously discovered potentially high-performance fermentation yeast named IS258, and preliminary identification of its ethanol producing capability using un-optimized media (Simbolon et al., 2018), however its actual maximum ethanol yield and genetic identity is yet to be identified. Thus, this research was focused to study and optimize its fermentation media to discover its maximum ethanol yield using rich media from peptone, yeast extract, and glucose, and then to conduct DNA analysis to determine the species of the IS258 yeast. In this study we focused on optimizing the ethanol yield produced by the IS258 yeast based from the fermentation medium which previously used (Simbolon et al., 2018), which was further refined in this work using Response Surface Methodology (RSM) to uncover the yeast's true maximum potential for ethanol production. To confirm the genetic identity of the IS258 yeast, DNA sequencing was conducted. Lastly, considering this microbe good result in fermentation in non-aseptic environment and moderately high tropical climate upon fermentation, it holds great promise as high-performance bioethanol fermentation starter for future application.

MATERIALS & METHODS

Growth and Fermentation Media Preparation

Growth media for IS258 yeast propagation was made from 7.5g/L of peptone (Himedia), 4.5g/L of yeast extract (Himedia), 50g/L of glucose (Brataco). Peptone (7.5g) and yeast extract (4.5g) were mixed with sterile distilled water until the volume was 700mL, strirred using magnetic stirrer (MaxBlend) until homogeneous, and autoclaved at 121°C for 15 min (Daihan Scientific). Meanwhile, 50g of glucose was added distilled water until 300 mL, sealed and then was tyndallized at 90°C for 15 min (Precisdig), rested overnight at room temperature and repeated 3 times. Tyndallization method was used to avoid damage on glucose due to caramelization. Both solutions were then mixed in sterile condition prior usage inside steril laminar chamber (WINA), and pH was set to pH = 6.0 (Thermo Scientific). Fermentation media for RSM were made in 1,000mL volume and were similarly made, where compositions of peptone and yeast extract were made according to RSM treatment.

IS258 Yeast Sample

IS258 yeast that was previously isolated was stored in 1mL vial with 40% glycerol at -80°C. For every experiment, one vial of IS258 yeast isolate was rejuvenated by growing in 100mL sterile PYG liquid media at pH 6 (7.5g/L of peptone, 4.5g/L of yeast extract, 50g/L of glucose). IS258 yeast cells were grown at room temperature and shaken using a rotator shaker (Health) at 100rpm for 24 hours. The rejuvenated culture was then put into 900mL PYG media and for 24 hours in shaker rotator for further propagation. The yeast cell culture results from rejuvenation were collected by centrifugation (Herolab) at 5,000rpm for 5 min. The collected pellet of yeast cells was then washed with 0.85% NaCl (Merck) solution, precipitated using centrifugation (3,000rpm; 5min), and washing process were repeated 2 times. The pellets cells resulting from the washing process were then diluted using 0.85% NaCl solution and the cell density was adjusted at 660nm using spectrophotometer (Libra) = 5 (OD_{660}=5) and ready for use.

Ethanol Yield Optimization

Fermentation Media Composition Optimization

To maximize the ethanol yield of previously found IS258 isolate, the PYG media composition previously used for growth (Simbolon et al., 2018), was further optimized using Response Surface Methodology (RSM) model to obtain the optimum concentration of yeast extract and peptone composition, with the limiting factor in growth media is set to 20% glucose. The value of glucose content was set maximum at 20% because rarely natural sap sugar concentration will exceed that high concentration in the nature.

The experimental design used in this works was two-factor Central Composite Design (CCD). The optimized fermentation media compositions were developed from growth media PYG composition (Simbolon et al., 2018), which is 7.5g and 4.5g of peptone and yeast extract, respectively, which became the basis for determining the peptone-yeast extract combinations developed by RSM. The CCD value obtained was 1.414. Variables code to be tested: peptone and yeast extract, and coded levels can be seen in Table 1.

Table 1: Coded and corresponding values of Peptone and Yeast extract used in the RSM design.

Variable	Coded Levels				
	-1.414	-1.000	0.000	1.000	1.414
	Corresponding Values (gram)				
Peptone	5.38	6.00	7.50	9.00	9.62
Yeast Extract	2.38	3.00	4.50	6.00	6.62

From the CCD value as many as 13 combinations of yeast extract and peptone were obtained. Each of the combination was conducted with 3 replications, totalling 39 experimental fermentation units of fermentation were conducted. The variations summary of the different concentration of yeast extract and peptone actual combinations that were to be tested can be seen in Table 2.

Table 2: Variation of Peptone and Yeast extract variables used in this experiment

Variables		Variations	
		Peptone (gram)	Yeast Extract (gram)
Peptone	Yeast Extract		
0.00	0.00	7.50	4.50
+1.414	0.00	9.62	4.50
-1.00	-1.00	6.00	3.00
0.00	-1.414	7.50	2.38
+1.00	-1.00	9.00	3.00
0.00	0.00	7.50	4.50
0.00	0.00	7.50	4.50
0.00	0.00	7.50	4.50
-1.00	+1.00	6.00	6.00
-1.414	0.00	5.38	4.50
0.00	0.00	7.50	4.50
+1.00	+1.00	9.00	6.00
0.00	+1.414	7.50	6.62

The data obtained were analysed to obtain the RSM equation, where the shape surface response and to determine the general conditions of the experiment *Minitab 18* software was used. The coefficients on the empirical model are estimated with using multi-way regression analysis, where the coefficient of determination (R^2) was used to determine the suitability of the empirical model with the data experiment. D-optimal formulation was used to determine the composition of peptone and yeast extract to obtain maximum ethanol yield. As control experiment, 2 fermentation sets of 1,000mL of sterile PYG media, each with 7.5g/L peptone, 4.5g/L yeast extract, and 200g/L (20%) glucose are used, each with and without the addition of IS258 yeast starter culture. Control experiments of with and without addition of IS258 yeast starter culture, was designed to confirm the fermentation performance of IS258 starter culture that was used, and possibility of contamination in PYG media, respectively.

Fermentation and Distillation

The fermentation of various combinations of PYG media was carried out for each combination of PYG media, with 20% glucose in total of 1,000mL media for all 13 combination units. Fermentation was conducted in custom approximately 1050 mL glass fermentation container to minimize head space, with addition of 1% (*v/v*) of the IS258 yeast starter adjusted to OD_{660}=5 as fermentation starter into the fermentation container. The fermentation containers were equipped with ventilation hose which the output ends were inserted into metabisulphite solutions to venting the resulted CO_2 gas and prevent contamination. After addition of the starter, the fermentation containers were then sealed and stored at 28°C to continue fermentation. The fermentation process of 13 combination units of media compositions were carried out for 10 days and stirred twice daily at approximately every 12 hours. The fermentations were stopped for distillation after maximum of 10 days, despite observed gas which presumable CO_2 still visibly bubbling slowly approximately less than 1mL/min. The fermented media were distilled using two-stage distiller (Lab Bioin) and the obtained alcohol distillates from each tested combination was measured using alcoholmeter at 25°C which was calibrated previously with ethanol (Merck, *p.a*) and total ethanol content (ethanol concentration × distillate volume) and concentration (total ethanol ÷ volume) was calculated. This followed by Gas Chromatography (Shimadzu GC-2010) measurement for 5 highest ethanol concentration samples to confirm the ethanol results (Kim et al., 2022). The GC-2010 was equipped with FID detector and Restek TRX-5 column. Helium gas with 1.5mL/minute was used as carrier, and hydrogen (>99.99 purity) with atmospheric gas with flow 30µL/m and 300µL/m was used for combustion, respectively. The injector, oven, and detector temperature were set to 30, 80, and 180°C, respectively. The best media combination of peptone and yeast extract for the fermentation by IS258 yeast was determined from the highest ethanol yield.

DNA Barcoding – Species Identification

To identify the IS258 yeast species, partial DNA sequencing was performed. For the DNA extraction, Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research) was utilized. The primers used for PCR were generic primers, forward: 27F: 5′ –AGAGTTTGATCMTGGCTCAG– 3′ and reverse: 1492R Primer: 5′ – GGTTACCTTGTTACGACTT– 3′. The PCR mixture comprised of dd-H2O 237.5µL; MyTAC Red Mix (Bioline) 312.5µL; and each 25µL of 10µM 27F

Primer (forward), 10μM 1492R Primer (reverse), and the extracted DNA template. PCR was carried out over 35 cycles with an initial denaturation, and followed by a single final extension. Subsequently, the PCR product was subjected to bi-directional sequencing. The resulting DNA sequence data was then compared (via BLAST) to entries in the database available at https://blast.ncbi.nlm.nih.gov/Blast.cgi for species identification.

Scanning Electron Microscopy – Morphological Identification

To identify the morphology of the IS258 yeast, Scanning Electron Microscopy (SEM) analysis was conducted. The IS258 yeast cell pellet was collected, fixated, and imaged using simple protocol without osmium tetroxide (University of Gothenburg, 2021). Approximately 10nm of gold sputter was applied using DII-29030SCTR Smart Coater, and then coated IS258 yeast was imaged using JEOL JSM-IT200.

RESULTS

Fermentation Media Nutrient Optimization

After the fermentation process was finished, the PYG media must from each of the media composition was distilled, and the distillate ethanol concentration was measured. The total ethanol obtained and the concentration value after distillation from combination of yeast extract and peptone concentrations in 1,000mL media using 20% glucose as limiting factor, can be seen in Table 3 as follow. The highest ethanol concentration from the IS258 yeast isolate was found to be 10.8% (*v/v*), a quite significant improvement from previously obtained 9.65% (*v/v*) using un-optimized fermentation media (Simbolon et al., 2018).

Table 3: Ethanol results as response of the variations of Peptone and Yeast extract in fermentation media

Peptone (g/L)	Yeast Extract (g/L)	Total Ethanol $^{\delta}$ (*v/v*)	Concentration (%) Alcoholmeter (GC)
7.50	4.50	102.75	10.1
9.62	4.50	107.71	10.8* (10.4)
6.00	3.00	77.52	7.7
7.50	2.38	84.77	8.5
9.00	3.00	97.19	9.7
7.50	4.50	102.59	10.3 (10.9)
7.50	4.50	101.20	10.1 (10.3)
7.50	4.50	102.51	10.2 (9.9)
6.00	6.00	81.71	8.2
5.38	4.50	76.32	7.6
7.50	4.50	102.40	10.2
9.00	6.00	104.37	10.4 (10.5)
7.50	6.62	92.46	9.2

Denote the highest value; $^{\delta}$total ethanol = distillate volume × concentration from alcoholmeter

As can be seen from Table 3, the average total ethanol has the highest value in combination #2 with a yeast extract concentration of 4.5g and peptone of 9.62g, which was later determined using the response surface methodology (RSM). Meanwhile, control experiments resulted in no ethanol content and approximately 10% v/v, for without and with addition of IS258 yeast starter, respectively. The slight improvement of ethanol results from the control experiment in comparison with the previous result (Simbolon et al., 2018), may be attributed to the tyndallized glucose, instead of the sterilization used in previous work. The highest total ethanol value shown in Table 3 is data later further processed using the response surface methodology (RSM), so it is necessary to obtain the most optimal yest extract and peptone concentration values. RSM data processing uses the total ethanol average as a data source so that the regression value is obtained from *Minitab* using the regression equation model, namely:

Ethanol concentration = -13.79 + 3.935 Peptone + 2.701 Yeast extract - 0.2206 Peptone*Peptone - 0.2983Yeast*Yeast + 0.0222 Peptone*Yeast

The results of the RSM equation show that the peptone concentration in the media gave the greater effect on the total ethanol produced by IS258. This influence can be seen from the peptone coefficient which has a value of 3.935 compared to yeast extract coefficient which has a value of 2.701. The RSM calculations show that the coefficient of determination (R^2) = 99.67% which explains that the correlation between yeast extract and peptone to total ethanol is 99.67% while the remaining 0.33% is influenced by other factors that cannot be explained by the response. Then the lack of fit value was used to test the suitability of the model and giving p-value of 0.496, where if the lack of fit p-value is greater than the value of α = 5% indicates that the created model is valid, and thus the model could be considered able to predict the response correctly. The response surface plot graph of the influence of yeast extract and peptone in ethanol production can be seen in Fig. 1.

The response surface plot graph formed in Fig. 1 resembles an inverted parabola that forms the maximum response at the peak. The maximum value of the ethanol yield can be seen from the shape and color changes on the surface plot from dark green to white. The increase in the amount of ethanol produced by IS258 yeast using fermentation medium is directly proportional to the increase in the concentration both peptone and yeast extract until it reaches the optimal point (whitest colored peak). The dark green area on the graph shows the lowest ethanol result, with concentration of yeast extract and peptone is approximately 3 and 6g/L. While the optimal area is white where yeast extract concentration of 5g and a peptone concentration of 9.5g. The Contour Plot Graph from *Minitab* of the effect of peptone and yeast extract can be seen in Fig. 2 as follows, with black dots represent the testing points of peptone and yeast extract compositions:

Overall, the optimal circular line meeting on the dark green contour plot graph has a center area of peptone concentration of 7.5–9.5g and yeast extract concentration of 3.25–6.5g. This broad area is the optimal area for the growth medium for IS258 yeast isolate which the optimal point for growth media composition at peptone and yeast extract concentration approximately of 9 and 4.5g/L, respectively.

D-Optimal Fermentation Media Composition and Fermentation Result

However, since the Surface Plot has yet to determine the maximal point which will results the most optimal ethanol yield, thus the determination of the specific amount of peptone and yeast extract combination was done by determining the D-optimal point in Fig. 3.

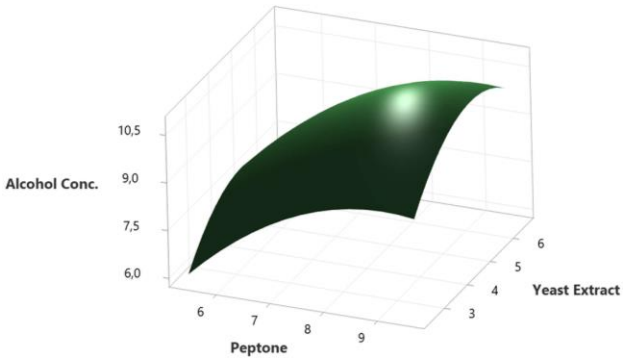

Fig. 1: Response surface plot on produced ethanol concentration yield (%) from various peptone (gram) and yeast extract (gram) combinations upon fermentation. *Image was created using Minitab.

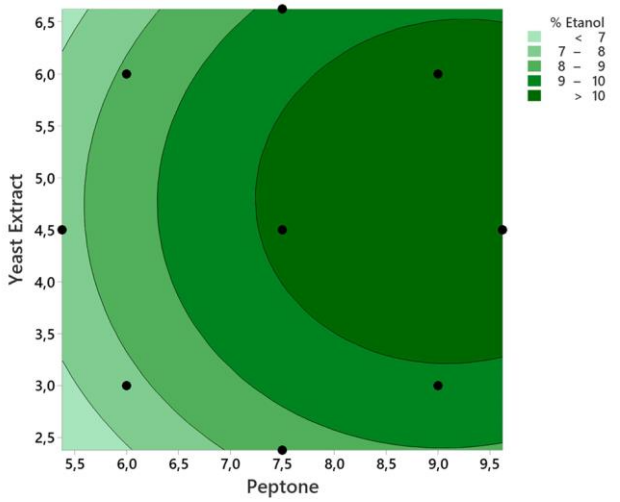

Fig. 2: Contour plot of ethanol concentration (%) results vs peptone (gram) and yeast extract (gram) combinations in fermentation media. *Image was created using Minitab.

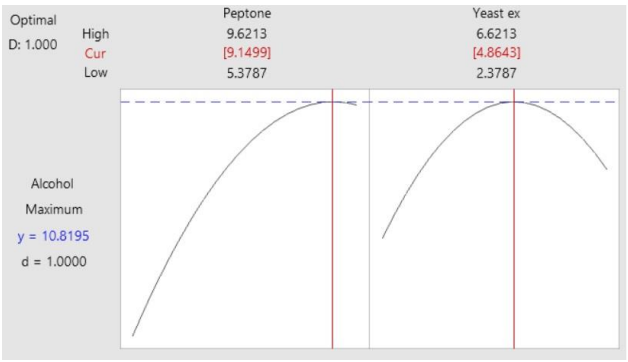

Fig. 3: D-optimal prediction of optimal ethanol concentration (%) yield from peptone (gram) and yeast extract (gram) in fermentation media. *Image was created using Minitab.

The D-optimal graph shows that IS258 requires higher amount of peptone in comparison with yeast extract, as could be seen from the Fig. 3 which shows concentration at 4.86g the yeast extract has reached its optimal limit. This likely indicates that further addition of yeast extract than 5g, will not increase the ethanol yield and further addition might potentially inhibits the yeast growth. Meanwhile for the peptone concentration, the optimal concentration was determined to be at 9.15g, where further addition of peptone will potentially show similar inhibition effect on the growth of isolates IS258. The result from D-optimal optimized value of fermentation media made from 9.15g of peptone and 4.9g, with similarly 20% glucose was tested, and would yield 10.82% of ethanol concentration as result. However, this result is not necessarily different than previously obtained maximum of 10.8% from RSM in Table 3 which indicates that IS258 can give similar ethanol yield results from quite broad peptone and yeast extract media compositions in the media. These indicate that it is possible in real-life application, IS258 will produce slightly over 10% ethanol despite the existence of slight variations of composition in saps used for fermentation media as long as essential nutrient for yeast growth is sufficient.

Distillate produced by IS258 upon fermentation was then submitted for compositional analysis using Gas Chromatography (GC-2010), with 5% (*v/v*) ethanol and 1% (*v/v*) methanol as standard solution to detect the presence of other volatile compound or methanol. From the GC analysis result of the distillate produced by IS258 in Fig. 4a, an existence of a peak which slightly lighter than methanol was observed in comparison with standard solution in Fig. 4b, this indicates that Mass Spectroscopy analysis was required to confirm the chemical composition of the compound whether if it is methanol. The presence of various small peaks which indicates the existence of traces of lighter compounds which is shown Table 4a, might originated from aromatic compounds produced by IS258 during fermentation which contribute to the distinct aroma of *Arak Bali* which is absent in standard solution as shown in Table 4b.

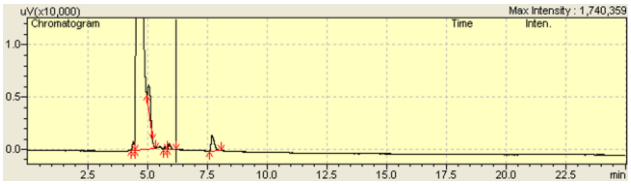

Fig. 4a: Chromatogram of IS258 distillate.

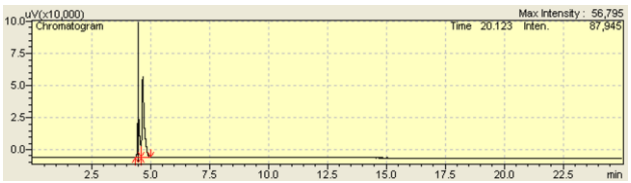

Fig. 4b: Chromatogram of 5% ethanol and 1% methanol (control solution).

Table 4a: Elution result of IS258 distillate

Peak#	Retention Time	Area	Height	Conc.	Units	Mark
1	4.412	5144.7	921.0	0.0000		
2	4.591	12545835.3	1738956.3	0.0000		sv
3	5.060	18502.3	2232.5	0.0000		t
4	5.731	1157.6	311.5	0.0000		
5	5.919	4563.5	679.3	0.0135	%	
6	7.703	15162.5	1512.3	0.0000		

Table 4b: Elution result of 5% ethanol and 1% methanol (control solution)

Peak#	Retention Time	Area	Height	Conc.	Units	Mark
1	4.488	215996.3	34956.6	0.0000		
2	4.679	420651.4	63151.5	0.0000		v

Genetic Analysis of IS258

Genomic DNA amplification by preparation using the Zymo extraction kit yielded approximately concentrations

of 45ng/μL and 30ng/μL of yeast (strain IS258) genomic DNA in both final volume of 30μL for subsequent sequencing (Table 5). The sequencing process was repeated twice to confirm the result, and to eliminate the possibility of contamination, and yielded DNA sequences of more than 600 base pairs for each attempt (Table 6).

Both of the obtained DNA sequences were matched in NCBI database (BLAST search: https://blast.ncbi.nlm.nih.gov/Blast.cgi) and consistent results were obtained. These both sequencing results show that IS258 belongs to the species *Nakaseomyces glabratus*, commonly referred to as *Candida glabrata*. Both sequences were submitted to NCBI SRA database with accession number: PRJNA1070026 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1070026). The *C. glabrata* is commonly found in fermented foods and has traditionally been considered non-harmful to humans. However, it was suggested that under certain conditions, it can act as an opportunistic pathogen (Fidel et al., 1999).

DISCUSSION

Fermentation Media Optimization

The RSM regression model gave R^2 value of 99.67, this suggested with high confidence that yeast extract and peptone in fermentation media plays crucial role in ethanol yield upon fermentation by IS258 yeast, at least in this work. Yeast extract in fermentation media contains amino acids and vitamin such as vitamins B which play an important role in supporting the activity and metabolism of microorganisms (Yun et al., 2003). In high concentration, the excess addition of yeast extract to the media will may be exceeds the optimal intake of IS258, and excessive yeast extract in media possibly will slightly increase total acid in the media upon fermentation (Pangestika et al., 2021). Higher acid levels will inhibit optimal yeast growth, since in general yeast has an optimum pH of 4.0–5.5 in order to grow optimally (Narendranath & Power, 2005). According to manufacturer (Himedia), peptone is rich in tryptophan content due to produce from digestion of animal tissue. Dilution of peptone in fermentation media will results pH of 7.2±0.2 at 25°C hence able to increase the pH of the fermentation media. Similarly, the addition of excess peptone will increase the medium pH that potentially would potentially inhibit growth of IS258 and affect the total ethanol yield. This helped to explain the inverted parabola shape of the response plot, where certain peptone and yeast extract yielded highest ethanol produce, and further increment of peptone and yeast extract concentration will simply reduce the ethanol yield possibly due to inhibition of yeast cell growth, or possibly even leading to yeast death. The broad optimal area (darkest green) in Fig. 2 also suggests the broad composition of nutrition content which IS258 will be able to utilize to produce optimum ethanol yield. However, the challenge would be finding the most efficient nutrition media which similar to the broad optimal area of yeast extract and peptone content obtained from RSM to efficiently and economically produce ethanol by IS258 yeast which will require further study in the future.

The actual and D-optimal calculation result suggest it would give 108mL of total ethanol, equal to 10.8% (*v/v*) ethanol concentration in 1,000 mL media. This result indicates approximately 84% sugar to ethanol conversion rate in comparison with ideal glucose to ethanol stoichiometric conversion, which is pretty good result considering the fermentation conditions is not yet fully optimized. However, addition of 1% (*v/v*) starter OD_{660}=5 with cell count approximately 5.32 x 10^{11} CFU/mL into the PYG media probably made adaptation and growth lag time by the IS258 was not required since already enough amount of cell density which probably does not required further propagation, thus high ethanol yield result. High starter cell density might enable IS258 to simply start the fermentation upon addition to media and deprivation of oxygen due to small head space in fermentation container, without the need of propagation hence enabled the high ethanol yield. This scenario might difficult to achieve in practical fermentation processes since addition of such high cell density to fermentation medium will not be financially and economically practical and will require better solution in the future for practical application. However, PYG rich media and high sugar content used in this works resulting in 84% ethanol from maximum

Table 5: Nucleic acid (genomic DNA) quantification (Nanodrop)

No.	Sample Name	Conc. (ng/μL)	$A_{260/280}$	$A_{260/230}$	Vol. (μg)	Date
1	IS258 seq attempt-1	45.5	1.87	0.82	30	28/09/2021
2	IS258 seq attempt-2	30.5	1.90	0.48	30	18/04/2022

Table 6: Sequencing results

Sample Name	Read DNA Sequences
IS258 seq attempt-1	CGGAGGAAAA GAAACCAACT GGGATTGCCT TAGTAACGGC GAGTGAAGCG GCAAAAGCTC AAATTTGAAA TCTGGTACCT TTGGTGCCCG AGTTGTAATT TGGAGAGTAC CACTTTGGGA CTGTACTTTG CCTATGTTCC TTGGAACAGG ACGTCATGGA GGGTGAGAAT CCCGTGTGGC GAGGGTGTCA GTTCTTTGTA AAGGGTGCTC GAAGAGTCGA GTTGTTTGGG AATGCAGCTC TAAGTGGGTG GTAAATTCCA TCTAAAGCTA AATACAGGCG AGAGACCGAT AGCGAACAAG TACAGTGATG GAAAGATGAA AAGAACTTTG AAAAGAGAGT GAAAAAGTAC GTGAAATTGT TGAAAGGGAA GGGCATTTGA TCAGACATGG TGTTTTGCGC CCCTTGCCTC TCGTGGGCTT GGGACTCTCG CAGCTCACTG GGCCAGCATC GGTTTTGGCG GCCGGAAAAA ACCTAGGGAA TGTGGCTCTG CGCCTCGGTG TAGAGTGTTA TAGCCCTGGG GAATACGGCC AGCCGGGACC GAGGACTGCG ATACTTGTTA TTCTAGGATG CTGGCATAAT GGTTATATGC CGCCCGTCTT GAACCAC
IS258 seq attempt-2	GCATATCAAA TAAGCGGAGG AAAAGAAACC AACTGGGATT GCCTTAGTAA GGGCGAGTGA AGCGGCAAAA GCTCAAATTT GAAATCTGGT ACCTTTGGTG CCCGAGTTGT AATTTGGAGA GTACCACTTT GGGACTGTAC TTTGCCTATG TTCCTTGGAA CAGGACGTCA TGGAGGGTGA GAATCCCGTG TGGCGAGGGT GTCAGTTCTT TGTAAAGGGT GCTCGAAGAG TCGAGTTGTT TGGGAATGCA GCTCTAAGTG GGTGGTAAAT TCCATCTAAA GCTAAATACA GGCGAGAGAC CGATAGCGAA CAAGTACAGT GATGGAAAGA TGAAAAGAAC TTTGAAAAGA GAGTGAAAAA GTACGTGAAA TTGTTGAAAG GGAAGGGCAT TTGATCAGAC ATGGTGTTTT GCGCCCCTTG CCTCTCGTGG GCTTGGGACT CTCGCAGCTC ACTGGGCCAG CATCGGTTTT GGCGGCCGGA AAAAACCTAG GGAATGTGGC TCTGCGCCTC GGTGTAGAGT GTTATAGCCC TGGGGAATAC GGCCAGCCGG GACCGAGGAC TGCGATACTT GTTATCTAGG ATGCTGGCAT AATGGTTATA TGCCGCCCGT CTTGAACCAC GGACCAA

theoretical yield is hoped to serves as result benchmark for future fermentation of nature-origin sugary biomass using IS258, in order to develop IS258 into tropical fermentation agent for locally available sugar.

DNA Barcoding and SEM Result

The DNA blast result determined the IS258 to be yeast-like *Nakaseomyces glabratus* (Takashima & Sugita, 2022), or more commonly known as *Candida glabrata*. This yeast-like fungus *C. glabrata* is known to be tolerant in environment with high lactate concentration (Zangl et al., 2020). Meanwhile, it was found out that *Tuak Bali*, a traditional fermented drink from coconut sap in which IS258 was previously discovered from, is rich in lactic acid which suspected due to the presence of lactic acid bacteria (Qonita et al., 2018), which might resulted from airborne contamination of *lau* upon drying in open air. This might be the clue on why *C. glabrata* is found to be the highest ethanol-producing microbe among several other candidates in the works by (Simbolon et al., 2018) instead of *Saccharomyces cerevisiae*. The *C. glabrata* simply able to survive better in comparison with *S. cerevisiae* in lactic acid condition (Zangl et al., 2020), and in non-aseptic *lau* making, harvesting and fermentation process of coconut sap where *Tuak Bali* was made, where lactic acid bacteria might contaminate the coconut sap making the fermented sap acidic. Thus, making the *C. glabrata* is better candidate for fermentation agent in sugary media contain lactic acid (Watanabe et al., 2008).

The ethanol-producing *C. glabrata* is also commonly found in fermented food in Indonesia (Sumerta & Kanti, 2017), Malaysia (Vincent et al., 2021) and Thailand (Limtong et al., 2002), which suggesting this ethanol-producing strain maybe prevalent in South East Asia. Previously isolated *C. glabrata* from Sarawak, Malaysia was also found to have high ethanol fermentation yield, approximately at 80% (Vincent et al., 2021), despite slightly higher theoretical yield was found in this work (84%) which probably due to higher cell count in fermentation starter.

Meanwhile, previously the *C. glabrata* was identified as potential opportunistic pathogen (Roetzer et al., 2011; Tam et al., 2015), candidemia disease which is known to be caused by *C. glabrata* was found to be mainly occurred in Northern America and Europe (Guinea, 2014). However, this locally-prevalent *C. glabrata* of IS258 yet to known to pose danger to the local population in Karangasem Bali, the place where it was isolated, probably because of the strength of the immune system due to daily exposure to candida fungi in daily life. It is could be speculated that from conducted genetic analysis, despite IS258 is definitely *Candida glabrata*, IS258 yeast might already adapted to local tropical environment where many sugar present in nature, since *C. glabrata* is crabtree positive (Kumar et al., 2019), which could be better utilize fermentation instead of respiration, prolonged adaptation to the sugar-based resourceful habitat in Bali Island which rich of saps and fruits, this might drove IS258 yeast to further develop efficient crabtree mechanism may be due to repression of mitochondria ability for respiration in genetic level (Zhou et al., 2024), thus reducing its pathogenicity. Despite this speculation, further study is needed to confirm the pathogenicity of IS258 yeast in the future.

The SEM analysis of the IS258 yeast pellet cells revealed similarly ovoid shape as previously observed *C. glabrata* sample as in (Monteiro et al., 2013). Simple application of glutaraldehyde as fixating agent, followed by dehydration process using series of gradually increasing ethanol concentration and then overnight freeze, and finally vacuum drying resulted in satisfactory image as can be seen in Fig. 5.

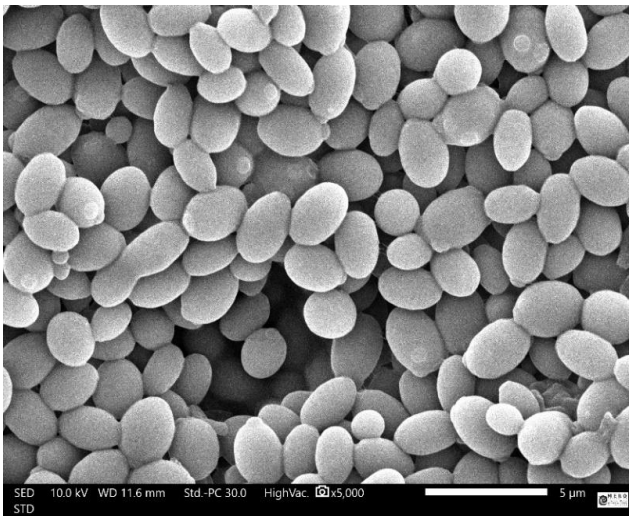

Fig. 5: SEM image of fixated IS258 pellet cells at 5,000 magnification.

IS258 Yeast Potential as Future Bioethanol Fermentation Agent

Previously, the IS258 yeast was found to have optimal fermentation temperature range of 26–28 °C (Prameshwari et al., 2024). The IS258 yeast with its tropical origin, might better to perform in tropical temperature range such as in Bali Island. With approximate conversion ability 84% of theoretical maximum yield in converting glucose into ethanol (Gombert & Maris, 2015), the IS258 as wild-type yeast has potential to become fermentation agent for industrial application in the future. However, in comparison with *S. cerevisiae* which able to produce with over 90% theoretical maximum yield (Della-Bianca et al., 2013), and comparison with commonly used *Saccharomyces cerevisiae* commercial strain, namely Ethanol Red and CAT-1, which commonly used in ethanol industries that can produced 1.57 and 1.49 mol/mol ethanol from glucose, respectively, with known ability of high-growth in moderately high temperature and high ethanol level (Lip et al., 2020; Silva et al., 2024), the IS258 yeast in this work was able to produced 1.65 mol/mol ethanol from glucose, which is promising despite the difference in fermentation methods. Considering IS258 yeast is wild type strain, its potentials may still can be improved in the future through further development using adaptive laboratory evolution (ALE), such as fermentation adaptation in high ethanol concentration (Mavrommati et al., 2023), higher fermentation temperature (Da Silva et al., 2022), or acidic condition (Salas-Navarrete et al., 2023), or possible bacteria-contaminated fermentation condition (Lino et al., 2021), which requires further research through stress-induced studies in order to develop IS258 yeast for better application in industrial level.

In this work, excess cells count was used upon starting the fermentation to fully obtain the highest ethanol yield without needs to lowering fermentation efficiency for cell propagation. Cell density of OD_{660}=5 at approximately 5.32 x 10^{11} CFU/mL measured by plate count method, which unlikely to be applicable in future practical applications. This difference in fermentation method may explain the high ethanol result, and the difference result obtained by (Lip et al., 2020; Silva et al., 2024). Thus, further improvements have to be made in the future regarding the ability of IS258 yeast to grow in industrial-grade fermenter with moderately high temperature, acidic condition, high ethanol concentration, and possibility of competing-bacteria contamination, and also the needs of high cell requirement at the start of fermentation to achieve higher ethanol yield. Possible solutions to be applied are stress-induced modification of IS258 characteristics through multi-generation adaptive evolution of IS258 yeast in challenging conditions, such as high temperature, acidic conditions, high ethanol concentration, and the possibility of competing bacteria contamination to induce adaptation, or random or engineered mutagenesis for better application in industry (Adebami et al., 2022). Meanwhile, for the needs of high cell amount at the start of fermentation to improve fermentation efficiency, immobilization of IS258 yeast in hard matrix such as alginate, polyvinyl alcohol (PVA), and composite encapsulation could be the appropriate solution (Chacón-Navarrete et al., 2021), where IS258 yeast cell could be re-used for subsequent fermentation batch, which does not require fresh cell at the start of fermentation, with advantage of high cell count availability at the start of every batch of fermentation where IS258 yeast already immobilized in matrices, that could serve to be source of propagation upon fermentation. Since available sugary materials commonly available locally in Indonesia is mostly in the form of sap, for example from sugarcane, nipa palm, arenga palm, and palmyra palm, then the immobilization of IS258 yeast in matrix would be promising method due to easiness of recollection of both liquid sap and hard matrices containing immobilized IS258 yeast. Further works are needed to improve IS258 yeast in order to maximise its characteristic and potential for commercial use of bioethanol fermentation starter in the future.

Conclusion

In this work, the ethanol fermentation capability and genetic identity of IS258 yeast was successfully discovered. The IS258 yeast can produce 84% of the maximum theoretical yield of glucose to ethanol fermentation, which is lower than that already established for *Saccharomyces cerevisiae*. This will serve as a benchmark to the extent to which IS258 yeast will be able to convert sugary materials into ethanol if other material is used for fermentation media, assuming both macro and micro nutrients are sufficiently provided. The IS258 yeast is identified as *Nakaseomyces glabrata* from DNA barcoding, previously known as Candida glabrata, and is commonly used in fermented foods in Southeast Asia, and only recently its high ethanol-producing capability was identified. Interestingly, as it is commonly used in fermentation-related foods and drinks, this yeast may not be as harmful to the local population, as previously reported pathogenicity. This may suggest that pathogenic and fermentation yeast are different strains, which, however, will require further genetic analysis in the future.

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Data Availability: All the data is available in the article

Ethics Statement: No ethical approval was required for this study as it involved only non-sentient organisms (*Nakaseomyces glabrata*) and did not involve human or animal subjects. All experimental procedures were conducted in accordance with standard laboratory protocols for microbial research (BSL-1).

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