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Bioethanol Production from Non-Conventional Yeasts *Wickerhamomyces anomalus* (*Pichia anomala*) and Detection of *ADH1* Gene

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ABSTRACT

Bioethanol is an organic compound resulted from the fermentation of sugar substrates by microorganisms which is used as alternative energy sources. During bioethanol fermentation yeast are exposed to various fermentation stresses, including temperature, osmotic, and oxidative stresess. Such conditions may decrease ethanol production. We previously isolated fermentation-stress tolerance yeast isolates from traditional Balinese beverages, identified as *Wickerhamomyces anomalus* BT2, BT5, and BT6. However no data available regarding the bioethanol production of those isolates. Our study indicates that these strains could utilize various sugar substrates (glucose, xylose, maltose, sucrose) in oxidative fermentative media. The highest value of substrate utilization efficiency following 48 hours fermentation was shown by BT6 on glucose (61.02%), BT 2 on xylose (55.44%) and maltose (60.90%). Measurement of ethanol production by Gas Chromatography showed that the strains were able to produce higher ethanol on the glucose substrate than other substrates. For instance, BT6 could produce the highest ethanol production (5.00 g/L) amongst strains tested by using glucose as substrate. Yet, the particular strains could only produce 0.30 g/L and 0.65 g/L by using xylose and maltose, respectively. For further genetic engineering purposes, we detected *ADH1* gene from all three isolates, with high homology to the alcohol dehydrogenase from *Saccharomyces cerevisiae*, *Geobacillus stearothermophilus* and *Pseudomonas aeruginosa*. Further strain development can be carried out targeting the *ADH1* gene, important for ethanol fermentation.

1. Introduction

Increased economic activity and national security cannot be separated from the elevating demand and consumption of energy sources. The use of fossil energy needs to be reduced to evade the negative effect of its use on the climate and environment. Thus, currently the development and the use of alternative energy source or new and renewable energy (NRE) are gaining more attention worldwide. However, recent report shows that the value of using new and renewable energy (NRE) as an alternative to fossil fuels in Indonesia is relatively low (Langer *et al.* 2021). Increasing the role of NRE and biofuels as optimal bioenergy sources is one of the Indonesia National Energy Policy targets. One of the alternative energy sources currently being developed is bioethanol (Maryana *et al.* 2021).

Bioethanol can be produced via microbial fermentation reaction utilizing various type of substrates. Sugar rich-type of substrates such as sugar cane and corn could deliver efficient fermentation reactions. Indeed, those substrates has been majorly used for commercial bioethanol production in Brazil and USA (Della-Bianca *et al.* 2013; Lopes *et al.* 2016). The bioethanol production by utilizing complex substrate such as lignocellulose is now being developed for more sustainable bioethanol production. Such production of bioethanol is commonly known as second generation of bioethanol productions (Ricardo *et al.* 2011; Branco *et al.* 2019; Watcharawipas *et al.* 2022). In addition to substrate,

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microbes play an important role in the bioethanol fermentation. Currently, bioethanol production on an industrial scale mainly uses the yeast *Saccharomyces cerevisiae* as the fermentation agent because it has a high fermentation rate and is resistant to relatively high ethanol levels (Azhar *et al.* 2017; Jacobus *et al.* 2021). However, challenges such as fermentation-related stresses during fermentation which inhibit yeast growth needs to be solved for more optimum bioethanol production.

Pichia spp., is one of the non-conventional yeast group that shows potential characteristics for bioethanol fermentation. Indeed, previous study reported that *P. kudriavzevii* 1P4 was tolerance towards high temperature (42°C) and could utilize pentose sugar (Ulya *et al.* 2021; Khotimah *et al.* 2023). Utilization of pentose sugar is important in the second generation of bioethanol productions using lignocellulose as substrate. Since, pentose is resulted from the hydrolysis reaction of raw lignocellulose, in addition to glucose (Koti *et al.* 2016). It is worth noting that yeast *S. cerevisiae* is not capable of using pentose as sugar source. Previous studies have reported the production of ethanol production by yeast *P. stipitis* (Bellido *et al.* 2011; Li *et al.* 2011; Koti *et al.* 2016), *P. kudriavzevii* (Astuti *et al.* 2018; Chamnipa *et al.* 2018; Pongcharoen *et al.* 2018; Khotimah *et al.* 2023), and *Wickerhamomyces anomalus* (*P. anomala*) (Atitallah *et al.* 2020) using various type of substrates including xylose, hemicellulose, and lignocellulose hydrolysates.

In the previous research, we have isolated the yeast *W. anomalus* from traditional alcoholic beverages, Brem Bali. Yeast *W. anomalus* with codes BT2, BT5, and BT6 exhibited important characteristics on stress tolerance against high-temperature, ethanol, and osmotic stresses (Lenka *et al.* 2021). To determine the potential fermentative activity of these three isolates, in this study, we evaluated the kinetics of ethanol fermentation using various monomeric sugars and detect the presence of *ADH1* gene on each isolate. *ADH1* gene encodes alcohol dehydrogenase enzyme which plays important role in bioethanol fermentation (Ståhlberg *et al.* 2008; Karaoğlan *et al.* 2020). Thus, further studies of the yeast strain development can be carried out in targeting this particular gene.

2. Materials and Methods

2.1. Fermentation Test Using Oxidative Fermentative Assay

The fermentation test was carried out by using previous method (Rahmadhani *et al.* 2022). Yeast *W. anomalus* BT2, BT5, and BT6 were cultured in Yeast Peptone Dextrose Broth (YPDB) media for 24 hours as the sub-culture. Each culture was then inoculated at starting OD_{600} = 0.1, 1 in Oxidative Fermentative medium using different carbon sources including glucose, maltose, sucrose, and xylose. Cultures were incubated at 30°C for 48 hours and alteration on the medium color from blue (basic pH) to yellow (acidic pH) was observed. The occurrence of yellow-colored medium indicated the presence of fermentation reaction using the designated carbon source.

2.2. Measurement of Sugar Consumption Rate

The sugar consumption rate was assayed using previous method (Ulya *et al.* 2021). The Yeast BT2, BT5, and BT6 were pre-cultured in YPD medium as starter culture for overnight to reach OD_{600} at 0.7-0.8. Starter culture 1% (v/v) were transferred to YPDB media supplemented with 2% selected sugar substrate (glucose, maltose, xylose). The concentration of the sugar consumption rate was measured every 8 hours for 48 hours using the DNS method (Miller 1959). Substrate consumption rate was then calculated using the formula:

Substrate consumption rate = ($\Delta S \times 100\%$) / S0

ΔS = substrate consumption = final [glucose] –initial [glucose]

The sugar content was measured using standard glucose curve

2.3. Bioethanol Production

Quantitation of bioethanol production was done by using previous method (Khotimah *et al.* 2023). Yeast cells were cultured in YPDB media with 2% selected sugar as carbon source (glucose, xylose, and maltose). Measurement of ethanol concentration was carried out using gas chromatography following 48 hours of fermentation at 30°C. The measured ethanol production kinetic parameters include ethanol yield (Yp/s), ethanol productivity (Qp) and fermentation efficiency using the following formula:

Yp/s	= P / ΔS
Qp	= P / Fermentation time
Fermentation efficiency	= (P × 100%) / Theoretical ethanol
P	= Ethanol concentration

2.4. Gas Chromatography Assay

For each sample, a volume of 1 µL was automatically injected onto the GC column using a split syringe. The concentration of ethanol was determined by using GC 17A Shimadzu Gas Chromatograph, as previously described by Rahmadhani *et al.* (2022). A Rt Qbond plus column was employed in the system at starting temperature of 40°C and raised to 160°C following the injection of sample, at a rate of 20°C/min. The temperature system was then raised to 200°C at a rate of 50°C/min and kept for 8 minutes. Nitrogen was applied as carrier gas and set for a flow rate of 28 ml/min and at a pressure of 61 kPa. Ethanol was used as standard to measure the ethanol content in the sample.

2.5. *ADH1* Gene Detection

The genomic DNA of yeast BT2, BT5 and BT6 was conducted by using Presto TM Mini gDNA yeast Kit (Geneaid, Taiwan) following manufactures protocol. Quantity and quality of gDNA was then determined by using Nanodrop 1,000 Spectrophotometer (Thermo Scientific). *ADH1* gene was amplified using PCR with the primer sequence of *ADH1*-forward (5'-GTTTTCCCTTCCACCAAGAACA-3') and *ADH1*-reverse (5'-ACAATCAT CTAAACCAGAGATAGCC-3') with KOD-FX Neo PCR kit (TOYOBO, Japan). PCR reaction was performed as follows: predenaturation at 94°C (5 minutes); denaturation at 94°C (30 seconds); annealing at 55°C (45 seconds); elongation at 72°C (1 minute and 45 seconds) with; post-elongation at 72°C (10 minutes). The PCR reaction was done in 35 cycles. Amplicon of 1,100 bp was then sequenced at Genetika Science Indonesia. Nucleotide sequence was then analysed for similarity and homology toward EMBI-EBI database by using BLASTX program.

3. Results

3.1. Fermentation Test Using Oxidative Fermentative

The results of the fermentation test on Oxidative Fermentation medium with various substrates after 48 hours showed all yeast strains could ferment all sugar sources. In addition, a significant reduction of pH value was found following fermentation. Indeed, final pH was found at 4 in xylose-containing medium, and 5 in sucrose and maltose-containing medium (Table 1).

3.2. Substrate Consumption Rate

Almost similar pattern of sugar consumption was found in three yeast isolates which used three different sugars as substrate. Almost half concentration of substrate was used by these three isolates within 48 hours of fermentation (Figure 1). The lowest consumption rate of sugar was found in isolate BT5 which was grown in xylose as carbon source (42.23±3.50%). Meanwhile, the highest rate of sugar consumption was found in isolate BT6 which used glucose as substrate (61.02±0.34%). Interestingly, isolate BT2 was more capable to use maltose (60.90±1.09%) than that of glucose (46.21±0.58%) and xylose (55.44±0.77%). In contrarily, isolate BT6 (61.02±0.34%) was more preferable in using glucose than that of xylose (45.08±0.77%) and maltose (43.25±3.09%) (Table 2). These data indicate that each yeast isolate has different preference on the sugar source as substrate.

Table 1. pH alteration in oxidative fermentative media with different carbon sources following inoculation of yeast *W. anomalus* BT2, BT5, BT6

Isolate	Starting pH	Final pH			
		Glucose	Sucrose	Maltose	Xyose
BT2	7	4	5	5	4
BT5	7	4	5	5	4
BT6	7	4	5	5	4

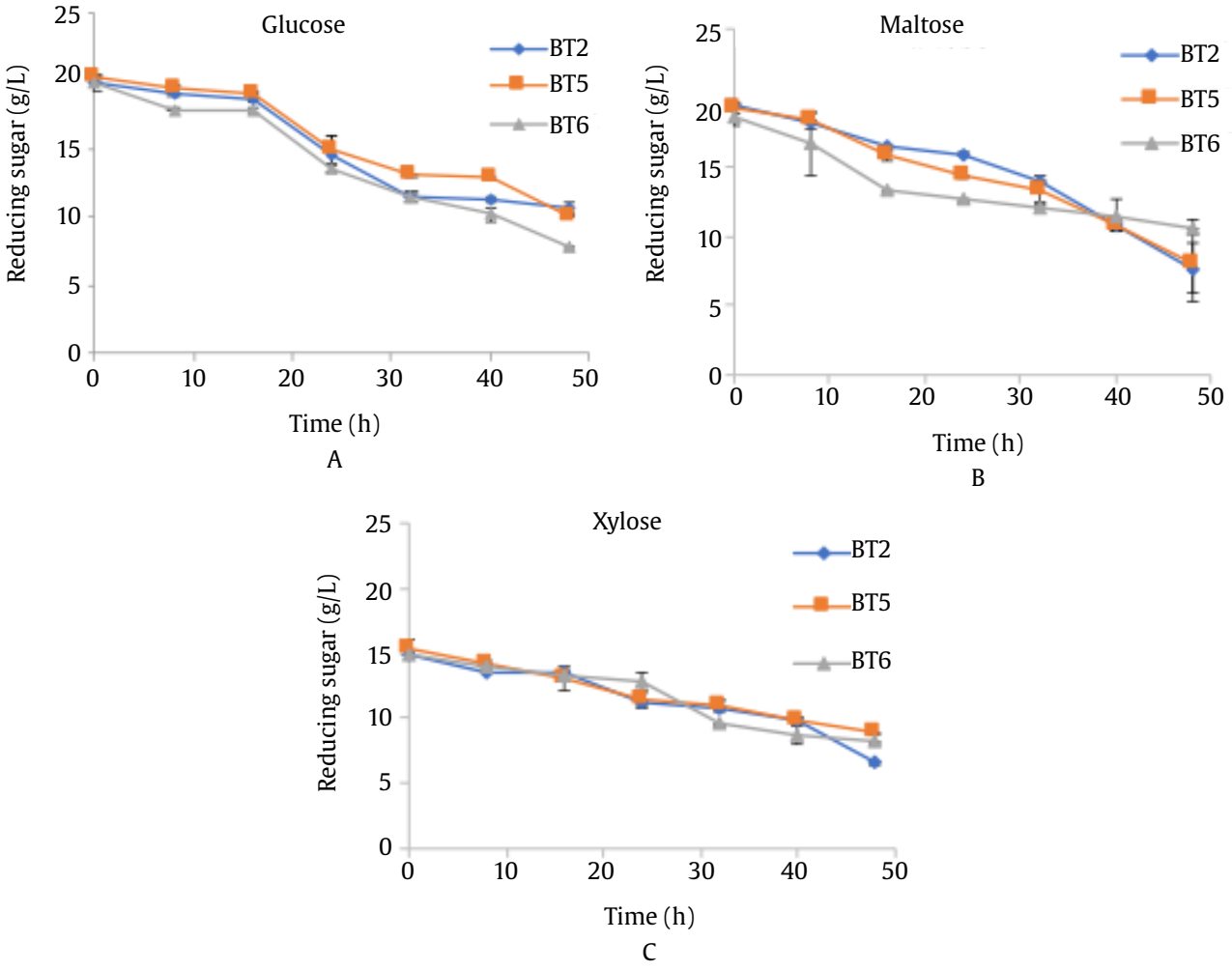

Figure 1. Sugar consumption pattern of yeast isolate BT2, BT5, and BT6 in different sugar compound as substrate (A) glucose, (B) maltose, and (C) xylose. The reducing sugar concentration was measured using DNS method, every 8 hours for 48 hours of incubation

Table 2. The substrate consumption rate (ΔS/s0) of three yeast isolates in different sugar as carbon sources

Substrate	Isolate	S0 (g/L)	S (g/L)	ΔS (g/L)	$\Delta S/s_0$ (%)
Glucose	BT2	19.81±0.62	10.65±0.36	9.16±0.25	46.21±0.58
	BT5	20.25±0.10	10.12±0.07	10.13±0.02	50.00±0.12
	BT6	19.87±0.02	7.74±0.07	12.12±0.05	61.02±0.34
Xylose	BT2	14.93±0.19	6.65±0.03	8.27±0.22	55.44±0.77
	BT5	15.40±0.70	8.89±0.13	6.50±0.84	42.23±3.50
	BT6	14.88±0.10	8.17±0.05	6.71±0.16	45.08±0.77
Maltose	BT2	19.59±0.07	7.66±1.81	11.93±0.22	60.90±1.09
	BT5	19.32±0.33	8.02±2.63	11.29±0.43	58.44±2.28
	BT6	18.75±0.77	10.63±0.53	8.11±0.47	43.25±3.09

(S0) initial sugar concentration, (S): final sugar concentration, (ΔS): sugar consumption, ($\Delta S/s_0$): sugar consumption rate

3.3. Fermentation Efficiency in Ethanol Production

The ethanol production of isolate BT2, BT5 and BT6 were found higher in glucose medium compared to that maltose and xylose after 48 hours of incubation (Figure 2). The highest ethanol kinetics was resulted by isolate BT6 in glucose as substrate with the ethanol production, ethanol productivity, and fermentation efficiency of 5 .00±0.42 g/L, 0.10±0.009 g/L/h, 80.61±6.84%, respectively. However, the ethanol productivity and fermentation efficiency were very low in the xylose and maltose as substrate, while xylose was found to be the least preferable carbon source for ethanol production (Table 3).

3.4. *ADH1* Gene Detection and Homology Analysis

Amplification of *ADH1* gene using PCR method resulted a 1,100 bp size of DNA target (Figure 3) from all three yeast *W. anomalus* isolates.

The homology analysis of DNA amplicon from isolate BT2, BT5 and BT6 showed the highest similarity (100%) towards *ADH1* gene sequence from *P. anomala* (AJ841789.2). Further homology analysis showed that decoded protein sequence of DNA amplicon of BT2, BT5 and BT6 were found homolog towards the crystalized protein of alcohol dehydrogenase 1 from *Saccharomyces cerevisiae* (76%), *Geobacillus*

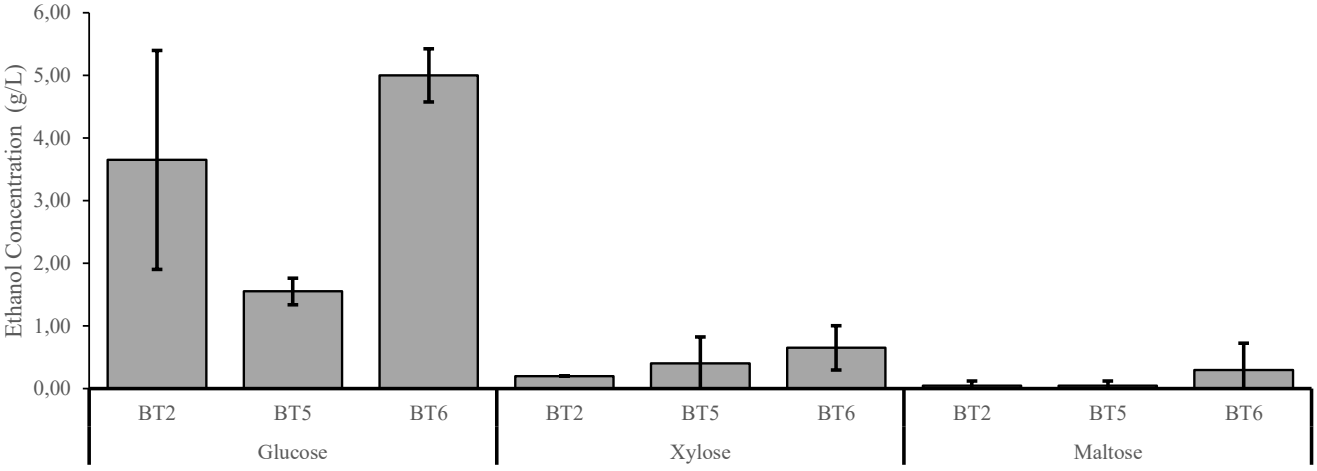

Figure 2. Ethanol concentration produced by three yeast *W. anomalus* isolates (BT2, BT5 and BT6) using different sugar compound (glucose, xylose and maltose) as carbon source after 48 hours of incubation at 30°C. Ethanol oncentration was measured using GC

Table 3. Kinetic parameters of ethanol production of *W. anomalus* isolates BT2, BT5, BT6 on selected substrates for 48 hours

Substrate	Isolate	P (g/L)	Qp (g/L/h)	Ey (%)
Glucose	BT2	3.65±3.75	0.08±0.078	77.99±80.08
	BT5	1.55±0.21	0.03±0.004	29.91±4.09
	BT6	5.00±0.42	0.10±0.009	80.61±6.84
Xylose	BT2	0.05±0.01	0.00±0.001	0.98±0.00
	BT5	0.05±0.42	0.00±0.009	1.25±10.63
	BT6	0.30±0.35	0.01±0.007	7.281±8.58
Maltose	BT2	0.20±0.07	0.00±0.001	6.23±2.20
	BT5	0.40±0.07	0.01±0.001	13.16±2.33
	BT6	0.65±0.42	0.01±0.009	29.78±19.43

(P): ethanol concentration, (Qp): ethanol productivity, (Ey): fermentation efficiency

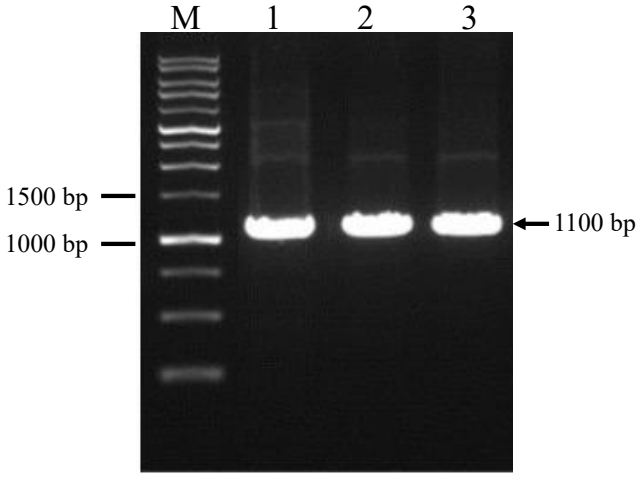

Figure 3. Amplicon of ADH1 (1,100 bp) from the genome of three yeast isolates resulted from PCR reactions. M : 1kb DNA marker, 1: BT2, 2: BT5, and 3: BT6

stearothermophilus (46%) and *Pseudomonas aeruginosa* (43%), respectively (Table 3).

4. Discussion

Ethanol fermentation employing non-conventional yeasts are gaining more interests currently. This is mainly due to enhance and develop more efficient and effective ethanol production to deliver new renewable energy of bioethanol in an affordable economical value. Indeed, non-conventional yeast such as *Kluveromyces marxianus* is recently being employed for production of bioethanol in a high temperature fermentation which support a significant reduction in cost production as compared to non-high temperature fermentation technology.

The yeast genera of *Pichia* spp. are one of the non-conventional yeast s that capable in using five-carbon source such as xylose (Domínguez *et al.* 2000; Martins *et al.* 2018; Martha *et al.* 2020; Ulya *et al.* 2021) . As confirmed in this research, yeast *W. anomalus* (*P. anomala*) BT2, BT5 and BT6 could utilize xylose as the main carbon source (Figure 1, Table 2). Such capability would benefit the utilization of this isolate in the second generation of bioethanol production employing lignocellulosic hydrolysate as the main substrate. In addition of glucose, xylose and arabinose are among the sugar compounds presence in the lignocellulosic hydrolysate (Luo *et al.* 2010; Srilekha Yadav *et al.* 2011; Oberoi *et al.* 2012; Ndaba *et al.* 2014). Thus, application of *Pichia* spp., which capable in using glucose and xylose would give essential enhancement on the fermentation process.

The substrate consumption rate of xylose and maltose by *W. anomalus* BT2, BT5 and BT6 were found at range 42 to 60% following 48 hours of incubation. Such range value was relatively similar to that glucose consumption rate (Table 3). Interestingly, the ethanol productivity and fermentation efficiency of these yeast isolates in xylose and maltose was significantly lower than that of glucose as substrate (Table 4). This might be due to the substrate consumption of xylose and maltose were designated for biomass production instead of ethanol synthesis via fermentation. Previous study reported the ethanol production might be stuck due to imbalance of carbon/nitrogen ratio. Indeed, the biomass yield of yeast *S. cerevisiae* was dependent on the amount of sugar concentration, availability of nitrogen and the nature of nitrogen source in the medium (Martinez-Moreno *et al.* 2012).

The isolate *W. anomalus* BT6 was capable to produce ethanol in glucose and was recorded as the most potential fermentative yeast in this study. The fermentation efficiency of isolate BT6 in glucose was found at 80%. This value is higher than previous studies of ethanol fermentation in yeast P. kudriavzevii (Ulya *et al.* 2021; Rahmadhani *et al.* 2022). The ethanol production and ethanol productivity of isolate BT6 was found at 5.00±0.42 g/L, and 0.10±0.009 g/L/h, from 2% of glucose, respectively. Such ethanol productions was relatively similar to the isolate *P. kudriavzevii* 1P4, which conducted ethanol fermentation using glucose at high temperature conditions (Ulya *et al.* 2021). Further strain development is essential to develop higher ethanol production in a mixed substrate of glucose and xylose.

Amongst the target genes for strain development of fermentative *Pichia* spp., *ADH1* gene is one of the potential gene for genetic engineering. *ADH1* encodes alcohol dehydrogenase enzyme plays essential role in ethanol fermentation reactions (Ståhlberg *et al.* 2008; Hasunuma *et al.* 2014; Karaoğlan *et al.* 2020). Our results indicate that all isolate of *W. anomalus* BT2, BT5 and BT6 have this particular gene which highly homolog toward those protein sequence of alcohol dehydrogenase. The fact that the homology of the protein sequence from yeasts BT2, BT5 and BT6 was aligned against the crystalized protein sequence, may give further benefit in the *in silico* study. Further analysis on the

Table 4. Homology analysis of protein sequence *ADH1* from *W. anomalus* BT2, BT5 and BT6 towards crystalized protein sequence

Yeast isolate	Species hit	Protein sequence homology	Identity (%)	E-Value	Region sequence	Accession number EMBI-EBI
BT2	*Saccharomyces cerevisiae*	Alcohol Dehydrogenase1 Closed with NADH	76	3.484e-176	1-347	7KC2.1
BT5	*Geobacillus stearothermophilus*	Alcohol dehydrogenase	46	8.343e-86	2-334	61QD
BT6	*Pseudomonas aeruginosa*	Alcohol dehydrogenase	43	3.204e-83	1-341	1LLU

modelling and docking assay would give valuable insight toward the potential target of *ADH1*-gene engineering that resulted mutant yeast with higher ethanol production than that of its wild type strains as well as common industrial-conventional yeasts.

In conclusion, yeast culture BT2, BT5, and BT6 were able to ferment various sugar substrates including glucose, sucrose, maltose, xylose, and dextrose. However, glucose is the most preferable substrate for bioethanol production. Isolate BT6 gave the highest ethanol fermentation kinetics, including ethanol production (5.00±0.42 g/L) and ethanol productivity (0.10±0.009 g/L/h), from 2% of glucose as substrate at 48 hours of fermentation. All yeast isolates have *ADH1* gene with high homology towards alcohol dehydrogenase crystalized protein from yeast *S. cerevisiae, Geobacillus stearothermophilus* and *Pseudomonas aeruginosa*.

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