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Enhancement of Isobutanol and 3-Methyl-1-Butanol Production Yields in *Saccharomyces Cerevisiae* without Genetic Modification

Nor Azah Ramli, Roshanida A. Rahman*, Rosli Md. Illias

Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

**Corresponding author: roshanida@cheme.utm.my*

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Abstract

Bio-based fuel produced from the renewable resources is efficiently overcome the shortcomings of fossil fuels. Several factors such as the increasing awareness on environmental problems, fossil fuel prices and the sustainability of energy has encouraged the initiative in finding another source of transportation fuels. Higher alcohols have proved to be a better candidate to replace gasoline as vehicle fuel due to characteristics of higher energy content, low solubility in water, lower vapor pressure and higher blending ability with gasoline. Biologically, isobutanol and 3-methyl-1-butanol are produced through the fermentation of renewable feedstock with microorganism. Saccharomyces cerevisiae is known to be able to produce isobutanol and 3-methyl-1-butanol titers naturally without heterologous pathways. However, the production of these alcohols by Saccharomyces cerevisiae is only in a small quantity, thus several efforts in enhancing the isobutanol and 3-methyl-1-butanol yields have been conducted. In this study, the amino acids (valine and leucine) and amino acid precursor (2-ketoisovalerate) were added into the fermentation medium prior to the fermentation. The results obtained show that the supplementation of 2-ketoisovalerate and leucine individually into the fermentation broth leads to the increased in isobutanol and 3-methyl-1-butanol titers by 3.3 folds and 1.9 folds, respectively. The combination of 2-ketoisovalerate and valine increased the isobutanol yield by 4.3 folds while the 3-methyl-1-butanol was increased by 2.5 folds when supplemented with 2-ketoisovalerate and leucine. These results portray that the isobutanol and 3-methyl-1-butanol titers can be improved by manipulating several factors which is important for future production of higher alcohols.

Keywords: Biofuel; isobutanol; 3-methyl-1-butanol; *saccharomyces cerevisiae*; Ehrlich pathway

1.0 INTRODUCTION

The growing concern on environmental problems and the decreasing of fossil fuel reserves ignites the production of liquid biofuel from renewable resources. Besides, the interest in replacing fossil fuel with biofuel is heightened due to the increased in global energy demand as well as the rising crude oil price [1,2,3]. Fossil fuel is a result of the decompositions of organic matters in the Earth. The use of fossil fuel as transportation fuel is known to be one of the major contributions in global warming as fossil fuel produce greenhouse gases such as methane (CH4) and nitrous oxide (N2O) as well as raises the atmospheric concentration of harmful carbon dioxide (CO2) [4]. The dependency on fossil fuel as energy sources for the continuous rising of transportation and industrial sector lead to the depletion of fossil fuel supplies. This situation results in the increase of the oil prices due to the fossil fuel supplies that cannot meet the energy demand in the future [5,6], directly affects the economic development worldwide. In addition, the production of biofuel is promoted by policies and regulations in several countries. The Europion Union for example has set criteria in using the biofuel as transportation fuel and also the usage of bioliquids as electric supplies in order to save the carbon and protects the biodiversity [7]. In Brazil, half of the energy supplied comes from renewable resources [8].

Biofuel is a renewable alternative to fossil fuel predominantly produced by renewable feedstock through fermentation of microorganisms [9]. Biofuel is considered the most sustainable alternative to replace fossil fuels as it has the potential in reducing greenhouse gases [10] as well as provides the positive impact towards economy. At present, higher alcohols production as transportation fuel attracts worldwide attention. Higher alcohols such as 1-propanol, 1-butanol, isobutanol, 2-methyl-1-butanol (2-MB) and 3-methyl-1-butanol (3-MB) are known to have chemical properties that make them suitable to be used as liquid fuel compared to bioethanol [3,11]. Isobutanol is one of butanol isomers that contain four carbon structures while 3-MB has five carbon atoms. These branched-chain higher alcohols possess several characteristics which make them beneficial as transportation fuel. Isobutanol and 3-MB contain high octane value and high energy density that are comparable with gasoline [1]. In addition, higher alcohols also exhibits another advantages including suitable to be used in pure form or blended with gasoline to any concentration without engine modifications, less soluble in water and have lower vapour pressure compared to ethanol [12].

Baker's yeast, Saccharomyces cerevisiae is one of the most promising hosts for the production of biofuel [2]. Saccharomyces cerevisiae has the ability in producing a small amount of isobutanol and 3-MB naturally through the catabolism of amino acid in Ehrlich pathway during the fermentation process [13,14]. In alcohol fermentation, the usage of natural alcohol host gives an advantage which is the genetic modification method can be avoided. This genetic or metabolic modification method is a complicated process [15]. Saccharomyces cerevisiae is appropriate yeast to be used as fermentation host as it possess numerous advantages such as having high alcohol tolerance; enable to tolerate isobutanol titers up to concentration of 20 g/l [2]. Besides, Saccharomyces cerevisiae has high robustness as it is able to resist harsh conditions during fermentation and tolerant to low pH, resulting in low risk of contamination [16,17]. In addition, Saccharomyces cerevisiae is also known to have facultative characteristics thus the complex equipments and facilities are not required for the fermentation process.

Higher alcohols are produced by the degradation of amino acids in yeast through Ehrlich pathway. Figure 1 presents the biosynthesis pathway for the production of isobutanol and 3-MB. The glycolysis process in cytosol converts glucose to pyruvate before being transported into the mitochondria by mitochondrial pyruvate carriers (MPCs) [18]. In 2-ketoisovalerate (2-KIV) synthesis, pyruvate is decarboxylated and condensed by acetolactate synthase (Ilv2p) in order to produce 2-acetolactate. The acetohydroxyacid reductoisomerase (Ilv5p) is responsible in reducing the acetolactate to 2, 3-dihydroxy-isovalerate which is then converted to 2-KIV by dihydroxyacid dehydratase (Ilv3p) [19]. Valine can be synthesized by branched-chain amino acid transaminase either in mitochondria (Bat1p) or in cytosol (Bat2p). The 2-KIV in cytosol is decarboxylated by α-ketoacid decarboxylase (KDCs) and being reduced by alcohol dehydrogenase (ADHs) to produce isobutanol titers [20]. 2-KIV is also directly involved in the biosynthesis of leucine. 2-KIV as a leucine intermediate is being converted to 2-isopropylmalate (2-IPM) by 2-isopropylmalate synthase (Leu4) and then catalyzed by another two enzymatic steps involving isopropylmalate isomerase (Leu1) and 3-isopropylmalate dehydrogenase (Leu2) to 2-ketoisocaproate (2-KIC). 2-ketoisocaproate can be converted to 3-MB through decarboxylation to isoamylaldehyde by α-ketoacid decarboxylase (KDCs) and then reduced by alcohol dehydrogenase (ADHs) [14].

Basically, for enhancing the isobutanol and 3-MB titers, several researches have focused on the overexpression of related genes, re-localization of the pathway in the same compartment and deleting the genes that inhibited the product formation. The overexpression of genes including ILV2, ILV5, ILV3, BAT2, KDCs and ADHs in valine biosynthetic pathway have been conducted to increase the isobutanol levels [17,21,15]. The overexpression of genes in Saccharomyces cerevisiae produced 376.9 mg/l isobutanol and 765.7 mg/l 3-MB, 34 folds higher than the control strain (without genetic modification) [14]. The re-localization of valine biosynthesis in cytoplasm or the overexpression of KDCs and ADHs in the mitochondria enables to improve the isobutanol yield [13,21,22]. The expression of enzymes in Saccharomyces cerevisiae's mitochondria produces the highest isobutanol concentration of 279 mg/l (minimal media) and 635 mg/l (complete media) [22]. The deletion of BAT1 in Saccharomyces cerevisiae CEN.PK2-1C results in the increase of isobutanol titers by 14.2 folds. On the other hand, when the BAT2 is deleted the isobutanol yield remains approximately the same with the wild type [18].

Several techniques have been performed in order to enhance the isobutanol and 3-MB titers during fermentation. However, the process in enhancing the isobutanol and 3-MB involves complicated steps which are the modification of microbial genes through genetic and metabolic engineering. Based on the Ehrlich pathway, the production of isobutanol and 3-MB in Saccharomyces cerevisiae is directly related to amino acids. In order to increase the isobutanol and 3-MB production yield without genetic modification, several types of amino acids and amino acid precursor including valine, leucine and 2-KIV were added into the fermentation broth.

2.0 METHODOLOGY

This section presents the procedures used in this study. The experiment consists of several parts as shown below.

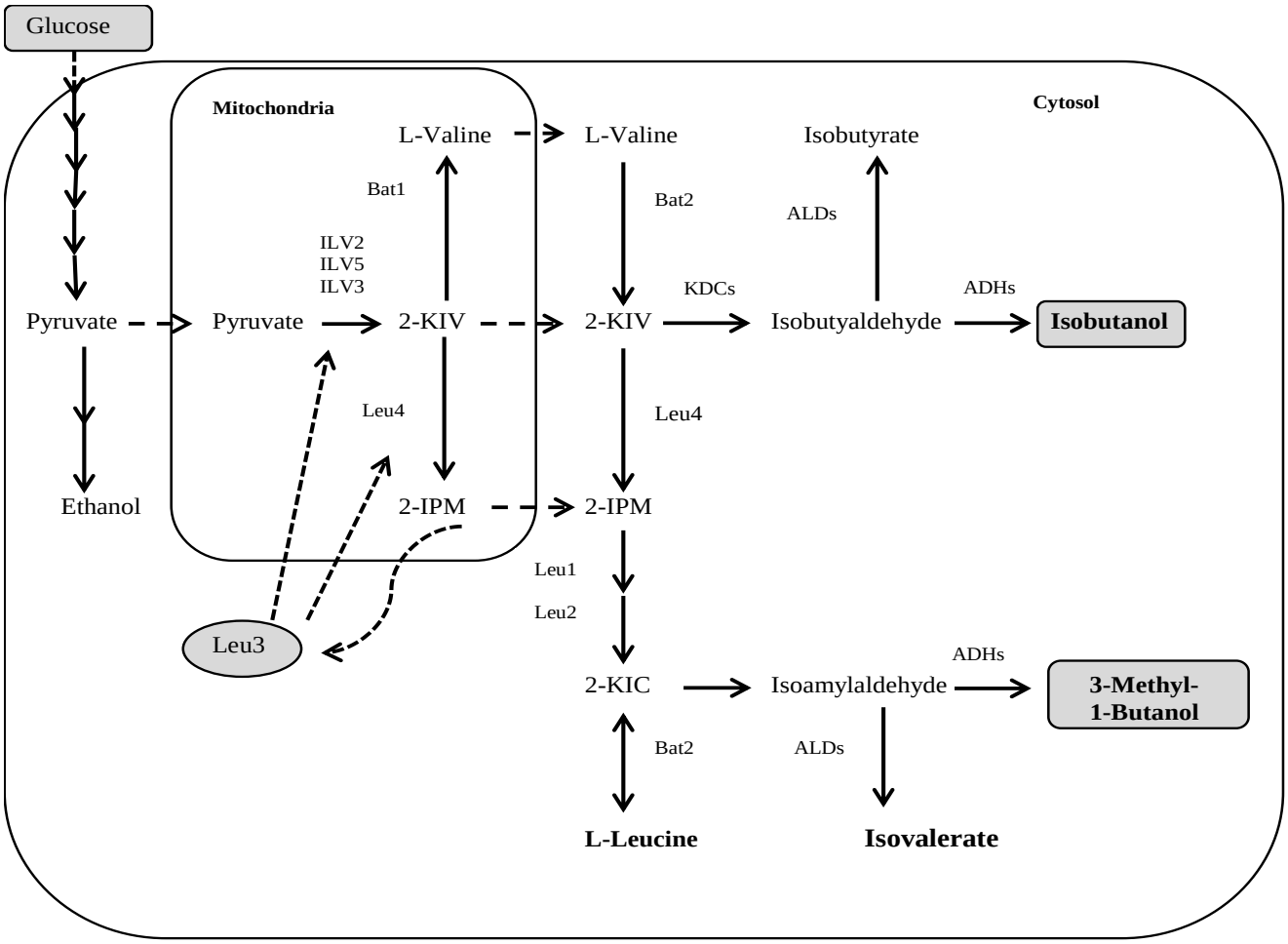

Figure 1 Schematic illustration of isobutanol and 3-methyl-1-butanol biosynthesis pathway produced by *Saccharomyces cerevisae* [14].

2.1 Strain and Inoculum Preparation

Saccharomyces cerevisiae was obtained from baker's yeast, Mauri-pan (AB Mauri Malaysia Sdn. Bhd., Malaysia). In order to prepare the inoculum, a loop of yeast cell that was grown on YPD agar for three days at 30 ± 0.5 ^{0}C was aerobically inoculated into 10 ml YPD medium broth and incubated for 20 – 24 hours at 30 ± 0.5 ^{0}C with orbital shaking at 170 rpm. The initial glucose concentration of the medium was 20 g/l.

2.2 Microbial Fermentation

The preculture (10 % v/v) was inoculated into 250 ml Erlenmeyer flask containing 50 ml medium (glucose (140 g/l), peptone (8 g/l), yeast extract (8 g/l), $(NH_4)_2SO_4$ (3 g/l), KH_2PO_4 (1 g/l), $MgSO_4.7H_2O$ (0.5 g/l) and $FeSO_4.7H_2O$ (0.05 g/l)). The carbon source was sterilized separately at 121 ^{0}C and added to the sterilized fermentation medium. The fermentation was cultivated at 28 ^{0}C in an incubator shaker with 179 rpm rotational speed for 48 hours. During the course of fermentation, samples were taken at 24 hours interval for analyses of isobutanol and 3-methyl-1-butanol. These experiments were conducted in triplicates.

2.3 Supplementation of Amino and Keto Acid

Before starting the fermentation, an amount of 0.5 to 1.5 g/l valine, leucine and 2-KIV were added individually into the shake flask containing the sterilized medium broth. The experiments were then followed with the addition of combination of 1.5 g/l of amino acids and amino acid precursor into the fermentation medium.

2.4 Gas Chromatographic Analysis

Fermentation products such as isobutanol and 3-MB were quantified by gas chromatography (Clarus 580 Perkin Elmer; USA) equipped with a flame ionization detector. The separation of alcohol compounds were carried out using a DB-WAX capillary column (30 m, 0.53 mm inside diameter, 0.5 µm film thickness). GC oven was initially held at 40 ^{0}C for 2 minutes and raised with a gradient of 15 ^{0}C / min until reaching 150 ^{0}C and held for 3 minutes. The injector and detector were maintained at 235 ^{0}C and 230 ^{0}C, respectively. Supernatant of culture broth were injected in split injection mode with a 20: 1 split ratio. Nitrogen was used as carrier gas and the combustion gas was a mixture of hydrogen and air.

3.0 RESULTS AND DISCUSSION

The results obtained were discussed in this chapter. The discussions were divided into two parts; the supplementation of valine, leucine and 2-KIV individually into the fermentation broth followed by the addition of the combined one.

3.1 Enhancing Isobutanol and 3-Methyl-1-butanol Production by Supplementation of Amino Acids and Amino Acid Precursor Individually

Two amino acids (valine and leucine) and one amino acid precursor (2-KIV) were supplemented into the fermentation medium broth in order to enhance the isobutanol and 3-MB titers in Saccharomyces cerevisiae. Figure 2 (a) presents isobutanol yields obtained after the addition of different concentration of valine, leucine and 2-KIV, individually. The addition of 0.05 g/l to 1.5 g/l 2-KIV resulted in the proportional increase in isobutanol titers. The highest isobutanol yield was 856 mg/l (using 1.5 g/l 2-KIV); 3.3 folds higher compared to the control yield (200 mg/l). The isobutanol biosynthesis pathway in Figure 3 shows that 2-KIV is the key in isobutanol production. The production of isobutanol in cytosol involves two steps mediated by several α-ketoacid decarboxylases (KDCs) and alcohol dehydrogenases (ADHs) [13]. It is likely that the increase in isobutanol production is a consequence of increase in catabolism of externally supplied 2-KIV into the cytosol.

The supplementation of valine in fermentation broth also increased the isobutanol produced by Saccharomyces cerevisiae. However, the concentration obtained was lower compared to the addition of 2-KIV. According to Figure 3, valine is synthesized in mitochondria from 2-KIV by Bat1 while the degradation of valine to 2-KIV occur in cytosol by Bat2. Based on the result, it can be predicted that the degradation of valine to 2-KIV in cytosol does not occur thus the isobutanol yield only enhanced in a small amount (further study has not been done).

Leucine gave the negative effect on isobutanol titers (Figure 2a). The isobutanol concentration decreased with the increased in leucine concentration in medium broth. Leucine, is one of the amino acid that leads to the 3-MB production during Ehrlich pathway in yeast strain thus the addition of this acid does not improve the isobutanol yield.

Figure 2 (b) depicts the 3-MB concentrations with the addition of amino acids and amino acid precursor. The addition of leucine into the fermentation medium caused the significant increase in the 3-MB levels. The highest yield (979 mg/l) was obtained with the addition of 1.5 g/l leucine. The titers increased by 1.9 folds compared to the yield without leucine supplementation. This result was expected, considering the mechanisms involved in the Ehrlich pathway [23]. Figure 1 shows that leucine is converted to 2-KIC in cytosol, the increase in leucine supplemented in the medium broth resulted in the significant increase in 3-MB concentration.

The addition of valine decreased the 3-MB in Saccharomyces cerevisiae. This is because valine leads to the production of isobutanol in Ehrlich pathway. On the other hand, the supplementation of 2-KIV during the fermentation process gave the small increment in 3-MB concentration. 2-KIV is an intermediate to leucine biosynthesis; requires three enzymatic steps to produce 2-KIC before being converted to 3-MB [14].

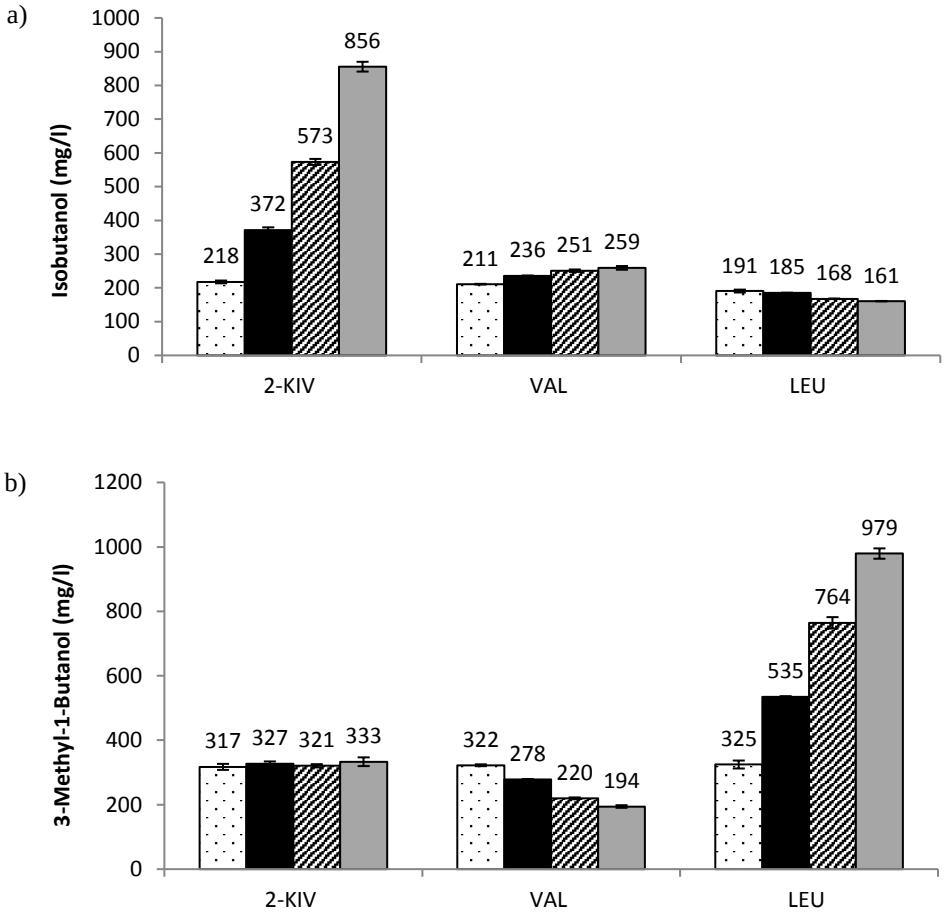

Figure 2 Effect of 2-ketoisovalerate, valine and leucine supplementation with different concentration on the higher alcohols production in *Saccharomyces cerevisiae* after 48 hours of fermentation. Figure 2 (a) shows the isobutanol production and Figure 2 (b) shows the 3-MB production. Error s represent the standard deviation of three independent fermentations. (Dotted bars) 0.05 g/l; (black bars) 0.5 g/l; (striped bars) 1.0 g/l; (grey bars) 1.5 g/l.

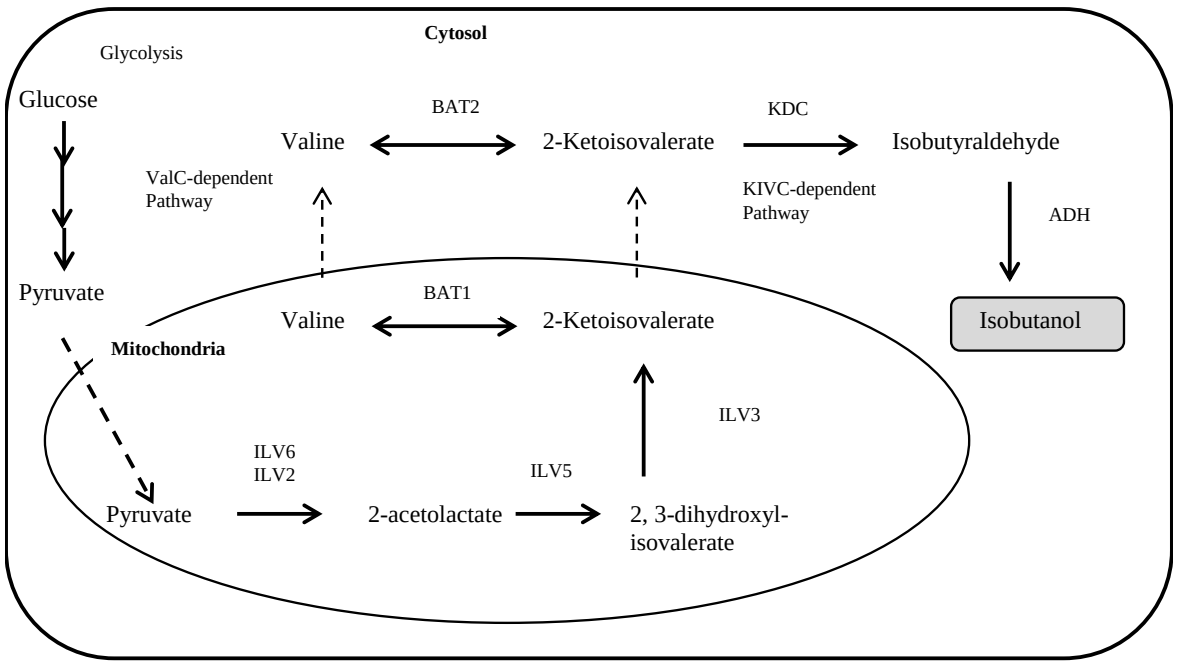

Figure 3 The biosynthesis of isobutanol naturally from glucose in *Saccharomyces cerevisiae*. Two possible pathways involved are ValC-dependent: valine transportation from mithocondria and KIVC-dependent: KIV transport from mitochondria [18].

3.2 Supplementation of Combination of Amino Acids and Amino Acid Precursor Mixture for the Isobutanol and 3-Methyl-1-Butanol Improvement

According to Procopio *et al.* (2015) [23], the higher alcohols increased with the addition of respective amino acids. This situation is achieved due to the increase in capacity to decarboxylate the transaminated α-keto acids and directly reduced to their respective higher alcohols [24,25]. Based on the result in Figure 2, the experiments were proceeded with the supplementation of 1.5 g/l amino acids and amino acid precursor's combination into the fermentation broth. Figure 4 presents the isobutanol and 3-MB concentration during the fermentation with combined 1.5 g/l amino acids and amino acid precursor. The combination of 2-KIV and valine gave the highest isobutanol concentration with 1058 mg/l, 4.3 folds higher than the control yield. 2-KIV and valine are directly involved in the formation of isobutanol in Ehrlich pathway (Figure 2), the supplementation of these nitrogen sources enables to improve the product titers. The highest 3-MB of 1178 mg/l results from the addition of 2-KIV and leucine. According to Figure 1, these amino acid and amino acid precursor lead to the 3-MB production. The increase in 2-KIV and leucine in cytosol enhances the production of 3-MB.

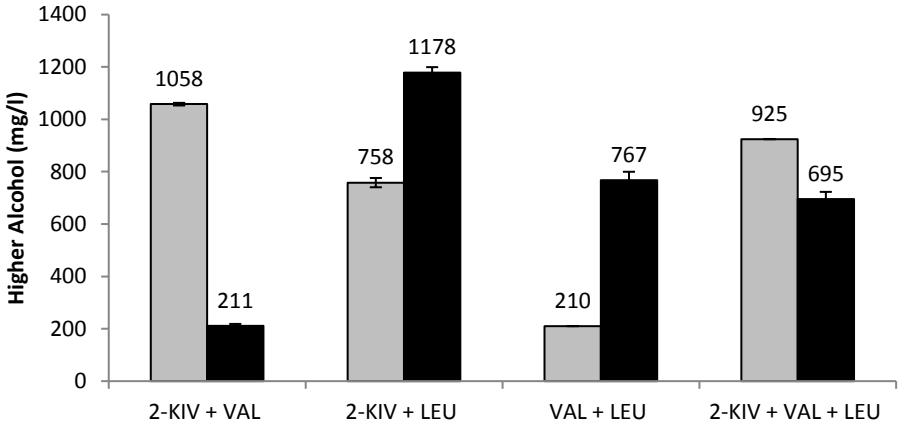

Figure 4 The production of (light grey bars) isobutanol and (black bars) 3-MB yield with the addition of 1.5 g/l amino acids and keto acid mixture. The titers were measured after 48 hours of fermentation. Error bars represent the standard deviation of three independent fermentations.

4.0 CONCLUSION

From the results of this study, it can be seen that the supplementation of amino acids and amino acid precursor is able to increase the higher alcohols production. Valine, 2-KIV and leucine are directly related to the isobutanol and 3-MB production in Saccharomyces cerevisiae through Ehrlich pathway. Therefore, the fermentation with addition of these nitrogen sources improved the isobutanol and 3-MB titers. The analysis in this paper shows that the higher alcohols titers can be improved by manipulating several factors without conducting the genetic modification on the microorganisms thus is important for the process improvement and future mass production of higher alcohols.

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