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Production of 3-Methyl-1-Butanol by Yeast Wild Strain

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***Abstract*—**The biomass-based fuels have become great concern in order to replace the petroleum-based fuels. Biofuels are a wide range of fuels referred to liquid, gas and solid fuels produced from biomass. Recently, higher chain alcohols such as 3-methyl-1-butanol and isobutanol have become a better candidate compared to bioethanol in order to replace gasoline as transportation fuel. Therefore, in this study, 3-methyl-1-butanol was produced through a fermentation process by yeast. Several types of yeast involved in this research including *Saccharomyces cerevisiae*, *Kluyveromyces lactis* GG799 and *Pichia pastoris* (KM71H, GS115 and X33). The result obtained showed that *K. lactis* GG799 gave the highest concentration of 3-methyl-1-butanol at 274 mg/l followed by *S. cerevisiae*, *P. pastoris* GS115, *P. pastoris* KM71H and *P. pastoris* X33 at 265 mg/l, 190 mg/l, 182 mg/l and 174 mg/l respectively. Based on the result, it proved that yeast have a potential in producing 3-methyl-1-butanol naturally.

***Keywords*—**Biofuel, fermentation, 3-methyl-1-butanol, yeast.

I. Introduction

THE demand of petroleum-based fuels has risen due to the increasing industrialization and motorization of the world [1]. Continuous depletion of petroleum fuel-reserves is one of the prime concerns in this fuel dependent era [2]. Besides, the environmental problem such as greenhouse effect, global warming and climate change area are also the issues to be resolved worldwide. Thus, there has been a global movement toward reduction usage of fossil resources [2], [3]. The interest in biofuel production has escalated dramatically in order to replace the petroleum-based fuels.

Bioethanol is two-carbon alcohol derived from the fermentation of biomass. It is commonly called ethanol when it is produced from petroleum-based feedstocks. Bioethanol is currently the most important renewable biofuel [4]. However, several physical characteristics such as low energy density, low vapor pressure which cause the cold engine difficult to start, miscible with water and toxic to ecosystems make it less attractive as biofuel compared to other higher alcohols [5].3-methyl-1-butanol is a five carbon alcohol that can be produced by fermentation of the biomass feedstocks with microorganism just like ethanol. 3-methyl-1-butanol or higher chain alcohols contain higher energy content compared to bioethanol thus the fuel consumption will be reduced and give a better mileage [6]. Besides, this fuel can be blended to any concentration with gasoline in internal combustion engines without engine modification. In addition, 3-methyl-1-butanol has higher energy density [7]) and lower vapor pressure than both bioethanol and gasoline [8], which results in lower levels of volatile organic compounds generated during handling [9].

Fungus and yeast are able to produce small amount of 3-methyl-1-butanol naturally through fermentation process [8]. The use of natural production host in solvent production offer great advantage; the use of heterologous pathway can be avoided [10]. Expression of alcohol synthesis genes is a complicated process yet only low yield titers produced. In addition, yeast is well known to be tolerant to alcohols. *S. cerevisiae* is able to grow in butanol concentration higher than 20 g/l [11]. Compared to bacteria, yeast is relatively robust thus resist to harsh conditions during fermentation process [10]. Most of the yeast has GRAS (generally regarded as safe) status, thus safe to be used in various application including food and feed applications.

References [12] and [13] have reported that *K. lactis* was able to produce 3-methyl-1-butanol naturally" while *S. cerevisiae* produced 3.4mM 3-methyl-1-butanol without gene expression [14]. In addition, *Pichia* species was reported as a good producer of ethanol and capable candidate for butanol production [15]. The objectives of this study were to identify the ability of these yeasts to produce 3-methyl-1-butanol naturally and to compare the yield produced in order to expose the best producer among the microorganisms involved.

II. Materials and Methods

A. Microorganisms

The wild strain of *Kluyveromyces lactis* GG799, *Saccharomyces cerevisiae* and *Pichia pastoris* KM71H, GS115 and X33 were obtained from Genetic Laboratory, Universiti Teknologi Malaysia (UTM). Each strain was maintained on YPD plate (glucose 20 g/l, yeast extract 10 g/l, peptone 20 g/l and 15 g/l agar) at 4^0C.

B. Inoculum Preparation

Liquid medium containing YPD medium (glucose 20 g/l, yeast extract 10 g/l and peptone 20 g/l) was used for inoculum preparation. Inoculum was performed aerobically in test tube containing 10 ml medium for 24 hours at 30 ^{0}C and 170 rpm in shaking incubator (Sastec).

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C. Microbial Fermentation

The preculture (5%) was inoculated into 250 ml flask containing 50 ml medium (40 g/l glucose, 8.33 g/l yeast extract, 8.33 g/l peptone, 8.33 g/l $(NH_4)_2SO_4$, 8.33 g/l KH_2PO_4 and 1.67 g/l $MgSO_4.7H_2O$). The carbon source was sterilized separately at 121^0C and added to the fermentation medium. The fermentation was cultivated at 30 ^{0}C in a shaking incubator with 170 rpm rotational speed for 96 hours. During the course of fermentation, samples were taken at 24 hours interval for analyses of 3-methyl-1-butanol, biomass and glucose.

III. Analytical Procedures

A. Biomass Concentration

The biomass concentration were determined by turbidity measurements at an absorbance of 660 nm (*K. lactis* GG799) and 600 nm (*S. cerevisiae* and *P. pastoris* species) using a UV/visible spectrophotometer (Jenway 6305 Spectrophotometer). Measured values were correlated to dry weight from duplicate samples that were centrifuged at 10000 rpm for 10 min, washed twice with distilled water and dried for at least 24 h at 80°C.

B. Reducing Sugar

The amount of carbon sources left in the samples after fermentation was determined using the dinitrosalicyclic acid method [16]. 1.5 ml of DNS reagent was added to 1.5 ml samples in test tubes. The tubes with the reaction mixture were heated in boiling bath exactly for 5 minutes and then cooled in ice water bath. The samples in tubes were diluted with distilled water (0.2 ml samples in 2.8 ml distilled water) and absorbance was measured at 540 nm wavelength. A blank sample was prepared with the same procedure using distilled water instead of problem sample [17]. Sample concentration was calculated based on calibration curve determined by using a known solution of glucose.

C. 3-Methyl-1-Butanol Determination

The 3-methyl-1-butanol produce was quantified by a gas chromatography (Perkin Elmer) equipped with a flame ionization detector. The separation of alcohol compound was carried out using a DB-WAX capillary column (50 m, 0.32 mm inside diameter, 0.5 µm film thickness). GC oven was initially held at 40 ^{0}C for 5 minutes and was raised with a gradient 10^0C/min until reaching 230^0C and held for 5 minutes. The injector and detector were maintained at 250^0C. Supernatant of culture broth was injected in split injection mode with a 1:15 split ratio.

IV. Results and Discussion

Fig. 1 illustrates the effect of yeast, namely *S.cerevisiae*, *K.lactis* GG799, *P. pastoris* KM71H, GS115 and X33 towards production of 3-methyl-1-butanol. The curve of 3-methyl-1-butanol production showing the high production occurred during exponential growth of yeast. As alcohol is the primary metabolite, it is directly involved in growth, development and production. *S. cerevisiae* and *P. pastoris* KM71H produced maximum concentration of 3-methyl-1-butanol when the fermentation time achieved 24 hours with 265 mg/l and 182 mg/l respectively. On the other hand, *K. lactis* GG799, *P. pastoris* GS115 and *P. pastoris* X33 were able to achieve the highest production yield during 48 hours fermentation with 274 mg/l, 190 mg/l and 174 mg/l.

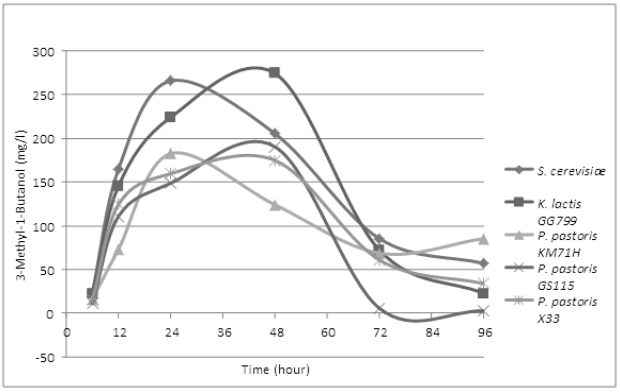

Fig. 1 Production of 3-methyl-1-butanol by *Saccharomyces cerevisiae*, *Kluyveromyces lactis* GG799, *Pichia pastoris* KM71H, *Pichia pastoris* GS115 and *Pichia pastoris* X33

Table I presents the 3-methyl-1-butanol production by several yeasts from previous studies. There are major differences quantitatively for 3-methyl-1-butanol yield by *K. lactis* in this study as compared with other study while for *S. cerevisiae*, the production of 3-methyl-1-butanol in this study was quite small compared to previous study. This situation might happen due to different in culture conditions, the medium composition and the strain itself.

TABLE I
PRODUCTION OF 3-METHYL-1-BUTANOL BY SEVERAL YEASTS

Microorganism	Production (mg/l)	References
K. lactis CBS 5670	10	[12]
K. lactis CBS 5670	73.33	[13]
K. marxianus	180	[18]
S. cerevisiae CEN.PK113-7D	299.71	[14]

The growth performance of yeasts was also determined as shown in Fig. 2. The exponential growth phase for all yeast started after 6 hours of fermentation. The yeast cell growth increased until achieved the stationary phase period between 50 and 80 hours of culture. The growth of *S. cerevisiae* was slightly lower compared to other yeast involved. This strain reached the maximum level of 10.6 g/l at the end of culture while the highest biomass concentration for *K. lactis* and *P. pastoris* (KM71H, GS115 and X33) were achieved at 72 hours of the incubation time with 13.2 g/l, 13.5 g/l, 13.7 g/l and 16.1 g/l respectively. "Reference [19] has performed a research on *K. lactis* and *S. cerevisiae* growth in aerobic condition". From the work, they have reported that *K. lactis* grew more efficiently than *S. cerevisiae*. This result supports

the classification of *K. lactis* as an aerobic respiratory yeast while *S. cerevisiae* as an aerobic fermentative yeast.

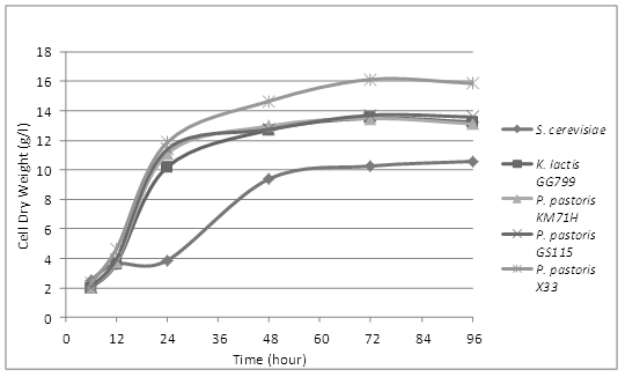

Fig. 2 Yeast cell growth during fermentation process of *Saccharomyces cerevisiae*, *Kluyveromyces lactis* GG799, *Pichia pastoris* KM71H, *Pichia pastoris* GS115 and *Pichia pastoris* X33 for 3-methyl-1-butanol production

Fig. 3 shows the changes in glucose concentration over time for five different types of yeast involved in 3-methyl-1-butanol production. As can be seen, there is an initial period of rapid consumption of glucose up to 24-48 hours, followed by a slower consumption period until the end of fermentation process in all strains. In this study, the 3-methyl-1-butanol fermentation was initiated with 40 g/l glucose in the medium and *S. cerevisiae* used 38 g/l glucose in only 24 hours of fermentation time. From the result, it was identified that *S. cerevisiae* was able to consume glucose rapidly, was about 18-22% higher as compared to other yeasts. "According to reference [20], *S. cerevisiae* was used in most industrial ethanol fermentations as it exhibits fast sugar consumption". Glucose consumption is important for the energy production which is used for the yeast survival and the energy produced leads to the production of 3-methyl-1-butanol.

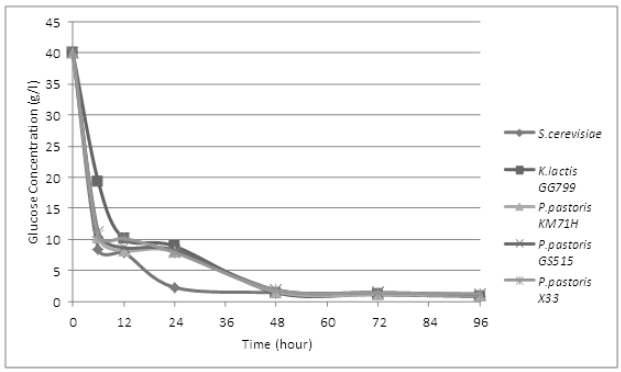

Fig. 3 Glucose consumption by yeast during fermentation process of *Saccharomyces cerevisiae*, *Kluyveromyces lactis* GG799, *Pichia pastoris* KM71H, *Pichia pastoris* GS115 and *Pichia pastoris* X33 for 3-methyl-1-butanol production

V. Conclusion

The results obtained production showed that all the yeasts used in this study were able to produce 3-methyl-1-butanol. *K. lactis* GG799 was identified as the preferred producer which able to produce the highest concentration of 3-methyl-1-butanol with 274 mg/l which was 3.4% higher than *S. cerevisiae* and 57% higher as compared to the poorest producer, *P. pastoris* X33 (174 mg/l).

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