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The Addition of Dextrose to the Preparation of Culture Media from Earthworm Juice (*Lumbricus rubellus*) for the Production of *Saccharomyces cerevisiae* FNCC 3012 Inoculum

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Saccharomyces cerevisiae is one of the commonly used yeast for bioethanol production. It can be produced by innoculation in a culture medium containing nitrogen and carbon. Earthworms (*Lumbricus rubellus*) are high in protein and have a relatively low price, which highly potential as source of nitrogen for *S. cerevisiae* FNCC 3012. The objectives of the study were to compare the growth rate of *S. cerevisiae* FNCC 3012 on earthworm juice (as natural media) and commercial media. Preparation of earthworm juice was carried out by boiling. Dextrose and sucrose were used as a carbon source. The growth rate of *S. cerevisiae* FNCC 3012 was measured by *optical density* (OD) and total reducing sugar (TRS). The results showed that earthworm juice with 1% dextrose addition produced higher yield of *S. cerevisiae* FNCC 3012 cells than commercial media. The maximum specific growth rate (μ_{max}) and the saturation constant (K_s) of dextrose were approximately at 3.4 h^{-1} and 3.1 g L^{-1}, respectively. In conclusion, earthworm juice can be valorised as a medium for production of *S. cerevisiae* FNCC 3012 inoculum.

Key words: cell yield, dextrose, earthworm juice, *Saccharomyces cerevisiae* FNCC 3012

Saccharomyces cerevisiae merupakan salah satu *yeast* yang biasa digunakan untuk produksi bioetanol. *S. cerevisiae* dapat diproduksi dengan menumbuhkannya pada media kultur yang mengandung nitrogen dan karbon. Cacing tanah (*Lumbricus rubellus*) mengandung protein tinggi dan memiliki harga yang relatif murah sehingga dapat digunakan sebagai sumber nitrogen untuk *S. cerevisiae* FNCC 3012. Tujuan penelitian adalah untuk mengetahui perbandingan tingkat pertumbuhan *S. cerevisiae* FNCC 3012 pada media jus cacing tanah dan media komersial. Pembuatan jus cacing tanah dilakukan dengan perebusan. Dekstrosa dan sukrosa digunakan sebagai sumber karbon. Tingkat pertumbuhan *S. cerevisiae* FNCC 3012 diperoleh dari pengukuran *optical density* (OD) dan total gula reduksi (TGR). Hasil menunjukkan bahwa media jus cacing tanah dengan penambahan dekstrosa 1% menghasilkan *yield* sel *S. cerevisiae* FNCC 3012 lebih tinggi dibandingkan media komersial. Kecepatan pertumbuhan spesifik maksimum (μ_{max}) dan konstanta saturasi (K_s) dekstrosa yang dihasilkan sebesar 3,4 jam^{-1} dan 3,1 g L^{-1}. Sebagai kesimpulan adalah jus cacing tanah berpotensi sebagai media untuk produksi inokulum *S. cerevisiae* FNCC 3012.

Kata kunci: dekstrosa, jus cacing tanah, *Saccharomyces cerevisiae* FNCC 3012, *yield* sel

Bioethanol is a renewable energy source that can replace fossil energy source (Zabed *et al*. 2014). Based on regulations issued by the Minister of Energy and Mineral Resources (Minister of Energy and Mineral Resources 2008) that premiums should be substituted with ethanol by 10% on 2020 and 15% on 2025. Bioethanol production depends on the use of yeast as a fermentation agent. *Saccharomyces cerevisiae* is one of the yeasts widely used for ethanol production (Sunaryanto *et al*. 2013). Based on data from Indonesian Statistics, it is predicted that 4,460.89 tons of active yeast will be imported in 2016 (Central Bureau of Statistics 2016). Thus, it is critical to improve the yeast production enabling to meet the increasing needs of yeast.

Yeast production can be carried out if there are media with sufficient nutrients (i.e. oxygen, hydrogen, carbon, nitrogen, vitamins, calcium, sulfur, minerals and water) for *S. cerevisiae* to grow (Hidayat *et al.* 2006). Peptone is one of the commercial nitrogen sources commonly used for *S. cerevisiae* (Batista *et al.* 2013). Indonesian Statistics stated that peptone are still imported, where its imports value still amounted to more than 4800 tons in 2016 (Central Bureau of Statistics 2016). Hajii *et al.* (2008) stated that the procurement of culture media can cost up to 40% of the overall cost of fermentation, while the price of commercial media is expensive (Arulanantham *et al.* 2012). Therefore, developing culture media using

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cheaper sources are critical.

Lumbricus rubellus contains crude protein at the value of 76% (Palungkun 2010). Protein content in other sources are as follows 62.5% in ram's waste (Kurbanoglu and Kurbanoglu 2002), 49.81% in fish meal (Mirzah 2008), and 19% in chicken bone residue (Wang *et al.* 2016). A high protein content in earthworms indicates that earthworms are potential to be used as a source of nutrients for yeast.

Earthworm extract contains lumbricin I compound (Cho *et al*. 1998), which has antimicrobial activity against pathogenic bacteria (Istiqomah *et al*. 2012), fungi and *S. cerevisiae* (Cho *et al.* 1998). However, this compound is easily damaged by heating (Hasyim *et al.* 2016). Therefore, heating was carried out in this study to eliminate the inhibition impact on the growth of *S. cerevisiae* FNCC 3012. The use of earthworm juice was expected to reduce the cost of purchasing substrate/media in fermentation industry, as well as the amount of imports for active yeast and peptone in Indonesia. The aims of this research were to valorise earthworm as a nitrogen source in yeast growth medium and to compare the growth rate of *S. cerevisiae* FNCC 3012 on earthworm juice and commercial media.

MATERIALS AND METHODS

Materials. This study used 2-month old earthworm (*Lumbricus rubellus*). Earthworm was obtained from CV. Rumah Alam Jaya (RAJ) Organic, Sukun, Malang. *Saccharomyces cerevisiae* FNCC 3012 was obtained from Center for Food and Nutrition Studies Universitas Gadjah Mada, Yogyakarta. The commercial media used include malt extract (Merck), meat extract (Merck), yeast extract (Merck), peptone (Oxoid). Dextrose and sucrose were used as carbon sources.

Culture Preparation. *S. cerevisiae* FNCC 3012 was grown in 100 mL sterile medium of Yeast Peptone Dextrose (YPD) (Batista and Fernandes 2015) and incubated for 24 h at 30°C (Schmacht *et al.* 2017). Then the culture of *S. cerevisiae* FNCC 3012 was measured using UV-VIS spectrophotometer (Thermo Scientific) at 600 nm wavelength (Batista *et al.* 2013) and calculated the viable cell using plate count method (Mailoa *et al*. 2017). The cells number obtained was 2.2 x 10^8 CFU mL^{-1} with optical density (OD_{600}) of 1.016. The unit cell count was then converted to mg mL^{-1} by multiplying the number of cells in CFU mL^{-1} by the weight of *S. cerevisiae* cells (3.70 x 10^{-8} mg $cell^{-1}$) (Barber and Lands 1973) and the water content of yeast (65%) (White 1952) to obtain wet cell weight of 5.692 x 10^{-8} mg mL^{-1}. Cultures are cultivated periodically on YPD agar medium.

Preparation of Earthworm Juice. Preparation of earthworm juice was based on the method used by Hidayat *et al.* (2017). A 150 g of cleaned earthworms (15% w v^{-1}) was blended with distilled water up to 1 L. The earthworm juice was stored in the freezer as stock solution. Take 150 mL earthworm juice, then it was boiled for 90 minutes using an electric stove with 600 Watt heat. The decoction of earthworm juice was filtered using Whatman 42 paper and sterilized at 121.1°C for 15 minutes.

Effect of Variations of Media and Carbon Sources Types. Experiments were conducted using the earthworm juice and commercial media. Each medium, with the volume of 20 mL, was added by 1% dextrose (w/v) and 1% sucrose (w/v) to compare how effective the growth of *S. cerevisiae* FNCC 3012 on different carbon sources. The media samples were inoculated with 20 µL (0.1%) culture (Batista and Fernandes 2015) and incubated for 24 h at 30°C. Then, the optical density (OD) of cells and total reducing sugar (TRS) were measured (Miller 1959). The medium samples with the highest cell yield was then selected to proceed the next step.

Effect of Variations of Dextrose Addition on the Earthworm Juice Medium. The 20 mL sterile earthworm was added with dextrose (1, 2, 3, 4, and 5%) (w/v) then inoculated with 20 µL suspension of *S. cerevisiae* FNCC 3012. These medium samples were incubated for 24 h at 30°C then measured the cell's OD and TRS. The values of specific growth rate (µ), maximum growth rate (μ_{max}), and saturation constant (K_s) were calculated using linear equations obtained from the value of specific growth rate per unit time. The data collected include the volume concentration of *S. cerevisiae* FNCC 3012 (X) biomass cells and the concentration of the reducing organic component (S). The kinetic parameter for determining the specific growth rate (µ) was calculated using equation 1a, the maximum specific rate of growth (μ_{max}) was calculated using equation 1b, and the cell yield on the substrate (Y x s^{-1}) was calculated using equation 1c (Dewi *et al.* 2016).

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (1a)$$

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}}\left(\frac{1}{S}\right) + \frac{1}{\mu_{max}} \quad (1b)$$

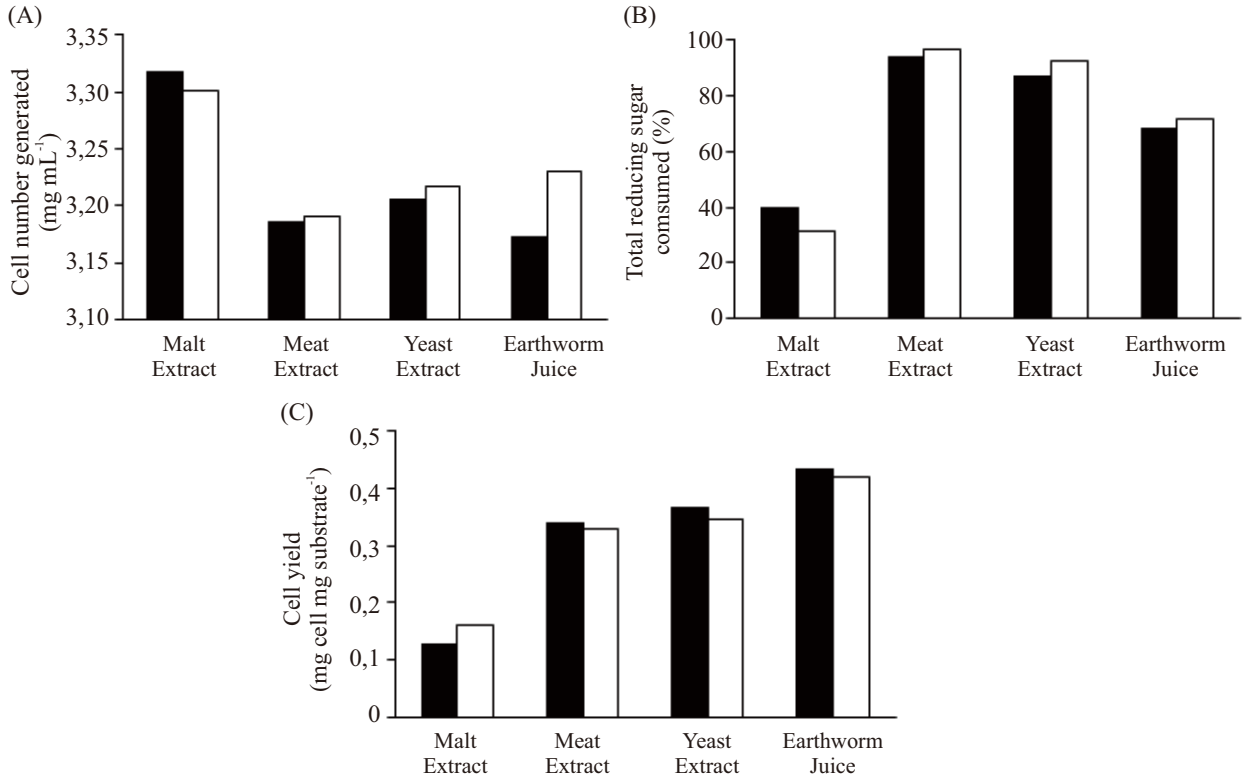

Fig 1 Cell number (mg mL^{-1})(A), TRS (%) (B), cell yield (mg cell mg $substrate^{-1}$)(C) on variations of medium and carbon sources. Symbols: (■) dextrose; (□) sucrose.

$$\frac{dS}{dt} = -\frac{1}{Y_{xs^{-1}}}\,\mu_{max}\left[\frac{S}{K_s + S}\right]X \qquad (1c)$$

RESULTS

The cells number of *S. cerevisiae* FNCC 3012 obtained by commercial media and earthworm juice were in the range of 3.172-3.317 mg mL^{-1} (Fig 1A). The number of cells from the highest to the lowest were produced by malt extract, earthworm juice, yeast extract, and meat extract, respectively. While, TRS consumed in meat extract, yeast extract, and earthworm juice were higher than that of the malt extract (Fig 1B). Cell yield of *S. cerevisiae* FNCC 3012 obtained by commercial media and earthworm juice were in the range of 0.129-0.431 mg cell mg $substrate^{-1}$ (Fig 1C). The highest to the lowest Y(x s^{-1}) values were obtained by earthworm juice, yeast extract, meat extract and malt extract, respectively. These findings indicated that the earthworm juice medium has a good potential for use as a growth medium of *S. cerevisiae* with the addition of 1% dextrose.

The experimental results demonstrated that dextrose addition at various percentages generated cells number of *S. cerevisiae* FNCC 3012 in the range of 3.173-3.565 mg mL^{-1} and the TRS consumed ranged from 68.5-84.7% (Fig 2A and 2B). The specific growth rate (μ) h^{-1} ranges was from 0.304 to 0.342 h^{-1} (Fig 3). The results also showed that increasing dextrose concentration causes a greater increase in the cell number, TRS consumed, and specific growth rate (μ) of *S. cerevisiae* FNCC 3012. The relationship between dextrose addition and specific growth rate was shown by the equation of y = 0.2967+0.928x (Fig 1). The results showed that the μ_{max} was 3.4 h^{-1} and K_s of dextrose was about 3.1 g L^{-1}. The yield cell of *S. cerevisiae* FNCC 3012 on earthworm juice medium ranged from 0.089 to 0.431 mg cell mg $substrate^{-1}$ (Fig 2C). The results, however, showed that as the concentration of dextrose increased, the yield cell value was decreased. The present work found that the highest yield cells *S. cerevisiae* FNCC 3012 was generated from earthworm juice with the addition of 1% dextrose.

DISCUSSION

S. cerevisiae utilizes nutrients in the medium to perform a series of biosynthetic processes to grow and produce new cells (Dewi *et al*. 2016). Amino acids as a source of nitrogen are essential for growth but high

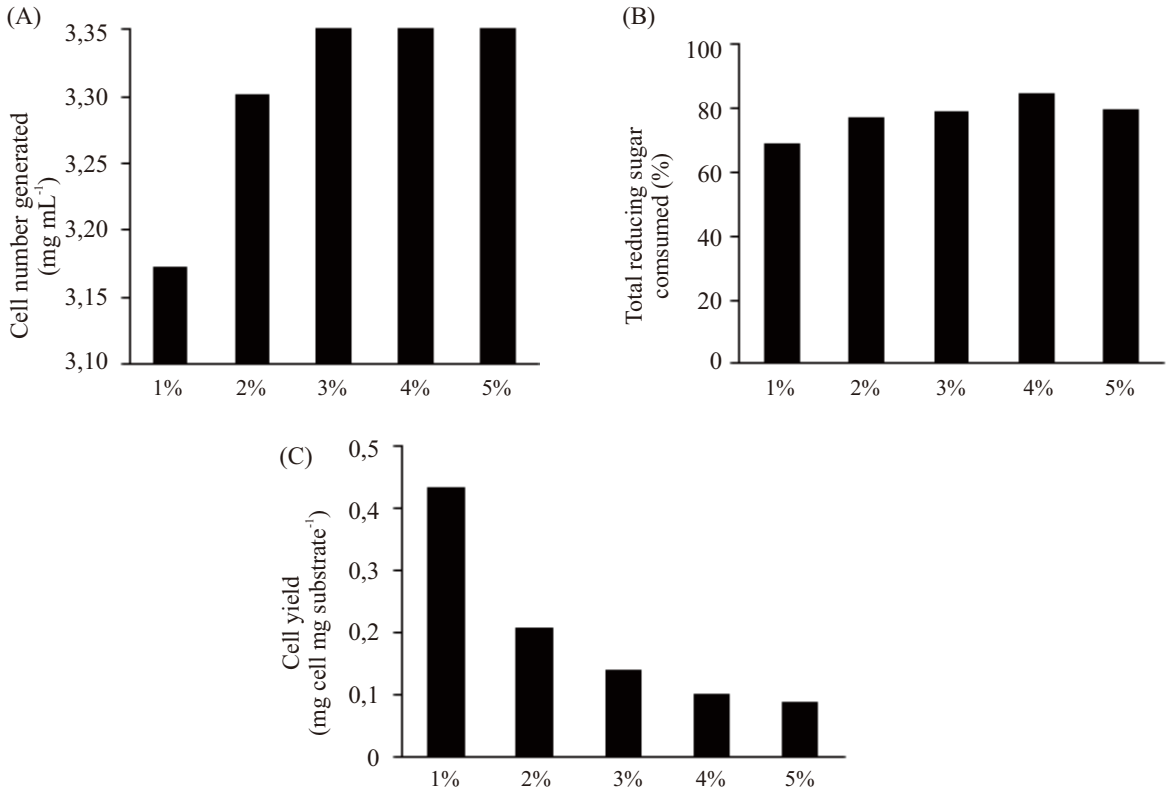

Fig 2 Cell number (mg mL^{-1}) (A), TRS (%) (B), cell yield (mg cell mg $substrate^{-1}$) (C) on earthworm juice with various percentage of dextrose. Symbols: (■) dextrose.

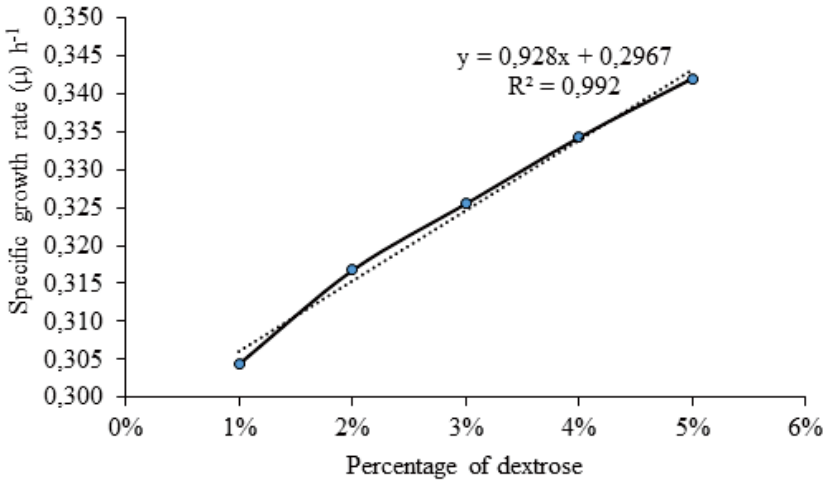

Fig 3 The relationship between dextrose addition and specific growth rate of *Saccharomyces cerevisiae* FNCC 3012.

intracellular amino acids become a toxic to the microorganism itself (Ruiz *et al.* 2017). The content of amino acid earthworm juice is still low enough, thus the nutritional needs of cells are possible supported by the presence of other nutrients than amino acids contained in earthworm juice and the dextrose addition as a carbon source.

The use of various types of media and carbon sources produced a diverse cells number of *S. cerevisiae* FNCC 3012 where the highest to the lowest cell numbers were produced by malt extract, earthworm juice, yeast extract, and meat extract, respectively. This difference is possible because cells also consume available sugars in the medium beside added dextrose or sucrose. Carbohydrates contained in malt extract, meat extract, and yeast extract were 80%,

Table 1 Amino acids compound on earthworm juice and commercial media

Amino Acids	Malt Extract [1]	Meat Extract [1]	Yeast Extract [1]	Earthworm Juice [2]
	mg kg^{-1}			
L-Aspartic	9000	75000	99000	992.00
L-Glutamic	16000	96000	163000	1326.00
L-Asparagine	-	-	-	71.33
L-Histidine	6000	19000	21000	242.00
L-Serine	4000	30000	46000	471.33
L-Glutamine	-	-	-	58.67
L-Threonine	4000	30000	43000	652.67
L-Glycine	4000	74000	48000	1416.00
L-Arginine	5000	38000	51000	488.00
L-Alanine	4000	54000	88000	3914.67
L-Tyrosine	3000	12000	24000	1582.67
L-Thryptophan +	2000	9000	14000	
L-Methionine	(no Trp)	(no Trp)	(no Trp)	1238.00
L-Valine	6000	48000	59000	3492.67
L-Phenylalanine	7000	37000	37000	1584.00
L-Isoleucine	5000	30000	55000	2569.33
L-Leucine	6000	60000	76000	2084.00
L-Lysine	6000	70000	80000	1340.00

Note: [1]ISO (2014), [2]Hidayat *et al.* (2017)

6.9%, and 7-13% (ISO 2014), respectively. Carbohydrates in a dry earthworm was 17% (Laverack 1963). Overall, TRS consumed in meat extract, yeast extract, and earthworm juice were higher than that of in malt extract. However, their cells number were lower than malt extract. Balcerek *et al.* (2016) and Pigman (1944) stated that malt extract contains α-amylase and β-amylase enzymes which hydrolyze the native starch in malt into maltose and glucose (Liao *et al.* 2010). The high carbohydrate content contained in malt extract (80%) will be hydrolyzed and can be utilized for *S. cerevisiae* growth so that the cells yield were high. Furthermore, the yeast extract does not produce an amylase enzyme (Liao *et al.* 2010) unless gene engineering has been performed to elicit the cell's ability to produce an amylase enzyme (Aydemir *et al.* 2014).

The highest cell yield of *S. cerevisiae* FNCC 3012 was produced by earthworm juice with dextrose addition of 1%, with the value of 0.431 mg cell mg substrate^{-1} higher than Win *et al.* (1996) of 0.23 g cell g sugar^{-1}. The cells yield on earthworm juice was high despite its lower amino acid content than that of in other media. It was possible because it has the amino acid Asparagine (Asn) and Glutamine (Gln) (Table 1) which is a type of amino acid rapidly absorbed by *S. cerevisiae* (Caballero *et al.* 2003). The findings in this study confirmed that the earthworm juice medium has a good potential for use as a growth medium of *S. cerevisiae* with 1% dextrose addition. This is in agreement with Brettel *et al.* (1980) who also reported that *S. cerevisiae* grew almost constant with levels of dextrose addition of 0.16-1.6%.

The specific growth rate maximum (μ_{max}) of 3.4 h^{-1}

was higher than the results reported by Manikandan *et al*. (2008) at value of 1.5 h^{-1}. The high of μ_{max} is possible because cell growth is triggered by the presence of nutrients such as minerals, amino acids or other growth factors (Hidayat *et al*. 2006) and other types of unknown sugar in earthworm juice other than added dextrose.

The value of K_s of dextrose obtained in this study was 3.1 g L^{-1}, which was lower than the dextrose from the banana peel of 25 g L^{-1} (Manikandan *et al.* 2008). The low of K_s is due to the high affinity of microorganisms to the substrate (Standbury and Whittaker 1984). Microorganisms with low K_s in a medium will produce higher growth rates than the microorganisms with higher K_s values (Arnaldos *et al.* 2015). According to Pramono *et al.* (2003), the lower of K_s value the better-used substrate. Interestingly, the value of K_s in this study is quite low, but the value of μ_{max} is higher than in other studies (Manikandan *et al*. 2008; Rorke and Kana, 2017; Singh and Sharma (2015); Voorhies 2012). It is possible that nutrients in earthworms can stimulate the growth of *S. cerevisiae* FNCC 3012. Those nutrients can be utilized by *S. cerevisiae* to form new cells (Chotineeranat *et al.* 2010). Hidayat *et al.* (2017) reported that there are 18 types of amino acids found in the earthworm (Table 1). Asn and Gln compound in earthworm may also have an effect on the values of μ_{max} and K_s. Asn is synthesized from aspartate, ammonia (or Gln), and ATP with the help of the enzyme Asn synthetase. The formation process of Asn involves glutamate and Gln (Moat *et al*. 2002), therefore the presence of Asn in earthworm can accelerate the tricarboxylic cycle and the rate of cell growth becomes high. Gln is a precursor for several other non-essential amino acids (Huang *et al*. 2017). Gln is synthesized from glutamate with the help of ammonia and ATP. The presence of Gln in earthworm can also accelerate the tricarboxylic acid cycle due to the cutting of the synthesis process of α-ketoglutarate to glutamate for Gln formation (Moat *et al.* 2002).

In conclusion this study revealed that the earthworm juice can be valorised for yeast growth medium. The results shown that earthworms were potential protein sources for inoculum production of bioethanol agents, especially *S. cerevisiae* FNCC 3012. A low cost of raw earthworm juice may added its potential for further scaling-up and commercialisation to fulfil the need of medium for inoculum production in Indonesia. Yet, more depth study is still urgently needed to improve its process and yield.

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