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THE POTENTIAL OF *Rhodotorula graminis* TISTR 5124 FOR SYNTHESIS OF POLYHYDROXYALKANOATE (PHA) BY LIMITATION OF A PHOSPHORUS AND NITROGEN SOURC

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ABSTRACT

Polyhydroxyalkanoate (PHA) is one of the alternatively biodegradable plastics which can be synthesized from a particular micro-organism after the fermentation process, considering the optimization of nutrients. In this research, the yeast strain *Rhodotorula graminis* TISTR 5124 was selected to be fermented with a carbon source in the standard nutrient in order to conduct a preliminarily study on the best conditions for this yeast in PHA production. The growth rate curve of yeast in the composition of imbalanced nutrients, i.e. the limitation of phosphorus and nitrogen, was also investigated and compared with another sample cultured in standard nutrients. Experimental results indicated that the condition that gave the maximum growth rate of this yeast strain was a P-limited condition at 81 hours, whereby the cell number of 3.1×10^9cells/mL was obtained and corresponded to the optical density (OD) of 0.95 measured at a wavelength of 600 nm. The synthesized PHA extracted from yeast cells after 81 hours of incubation was examined by Fourier transform infra-red (FT-IR) and nuclear magnetic resonance (^{1}H NMR) spectroscopy. The results indicated stretching vibrations similar to the copolymer PHBV (or a PHA derivative). Maximum PHA content of 54.4% was found in the P-limited condition which corresponded to a PHA yield of 65.1 (g/g-total sugar consumed) in which the yeast consumed the least glucose amount of 3.2 g/L, but grew the most rapidly. *Rhodotorula graminis* TISTR 5124 is therefore promising as a good candidate for alternatively biodegradable plastics, considering the potential to produce PHA and its derivatives. This process can be beneficial as an option to replace conventional plastics in the future.

Keywords: Bioplastic; Extraction; Polyhydroxyalkanoate; *Rhodotorula graminis*

1. INTRODUCTION

The use of non-biodegradable plastic leads to environmental problems and this situation has dramatically increased with the world's growing population. The production and combustion of conventional plastics create hazardous compounds, which are harmful to human health. Therefore, an idea to produce biodegradable plastics becomes very promising, due to the big challenge to replace the use of petroleum-derived plastics.

In recent years, three different categories of promising biodegradable plastics have been reported, which are known as: (1) chemically synthesized polymer; (2) starch-based biodegradable plastics; and (3) polyhydroxyalkanoate, PHA (Mose & Maranga, 2011;

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Suriyamongkol et al., 2007). The former has been shown to be susceptible to enzymes from microorganisms used during the fermentation. Thus, it cannot be commercially substituted for conventional plastics. Starch-based biodegradable plastics have proven partially degradable and have some drawbacks in regard to poor mechanical properties (Gaspar et al., 2005). Polymer obtained by microbial production such as PHA, proves to be degraded within a year under aerobic and anaerobic conditions using a variety of microorganisms.

Among these three types, PHA is an interesting choice and it has the potential to be produced by various microbes. However, the production cost of PHA from bacteria is high. Therefore, PHAs synthesized from yeast have become more interesting (Leaf et al., 1996). Although, the biochemical pathways of yeast are complicated, some authors have successfully engineered the monomer composition of PHAs in *Saccharomyces cerevisiae* with PHA accumulation up to 7% dry cell weight (Zhang et al., 2006). In this research, yeast *Rhodotorula graminis* TISTR 5124 was selected as a potential microbe to produce PHA and its derivative (homopolymer PHB and copolymer PHBV) because this species contributes to effective degradation of low molecular weight of aromatic hydrocarbon and very well-known in bioremediation (MacGillivray & Shiaris, 1993). When cells are grown under stressful conditions, i.e. an imbalance of nutrients, these cells usually convert the carbon source from sugar to PHA for energy storage (Poomipuk et al., 2014). PHAs are accumulated in the inclusion body (IB) and are specially produced rapidly when nitrogen and phosphorus in the nutrients are limited. This was found in various types of microbial bacteria, such as Bacillus and Azotobactor. The objective of this research is therefore to study the ability of *R.graminis* in the production of PHA under the limitation of phosphorus and nitrogen in the nutrients. The extracted PHA was examined by FTIR and NMR spectroscopy and compared the results with the standard copolymer.

2. MATERIALS AND METHODS

2.1. Yeast Strain and Nutrient Preparation

The yeast strain *Rhodotorula graminis* TISTR 5124 was used as received from the Thailand Institute of Scientific and Technological Research (TISTR) without any isolation. The culture was grown in 100 mL of broth at 30°C using a flask shaker speed of 180 rpm. The baseline condition is defined as Yeast Malt (YM) broth mixed with 0.9 g of $(NH_4)_2SO_4$ and 0.35 g of $NaNH_4HPO_4{\cdot}4H_2O$ as N and P source, respectively. In order to limit the N and P source, the amount of $(NH_4)_2SO_4$ and $NaNH_4HPO_4{\cdot}4H_2O$ decreased as follows in Table 1.

Table1 The amount of nutrient for PHA production in different conditions (in gram unit)

Nutrient	Baseline	P-limited	N-limited
Yeast extract	0.30	0.30	0.30
Malt extract	0.30	0.30	0.30
Peptone	0.50	0.50	0.50
Glucose	1.00	1.00	1.00
$(NH_4)_2SO_4$	0.90	0.90	-
$NaNH_4HPO_4{\cdot}4H_2O$	0.35	0.08	0.35

2.2. OD Measurement and Cell Number Count

Yeast samples at any interval were taken for OD measurement at 600 nm using an Ultraviolet Visible (UV-Vis) spectrophotometer (SPECORD 200). The cell numbers of the yeast in each sample was counted using a haemocytometer and visualized under the microscope as shown in Figure 1.

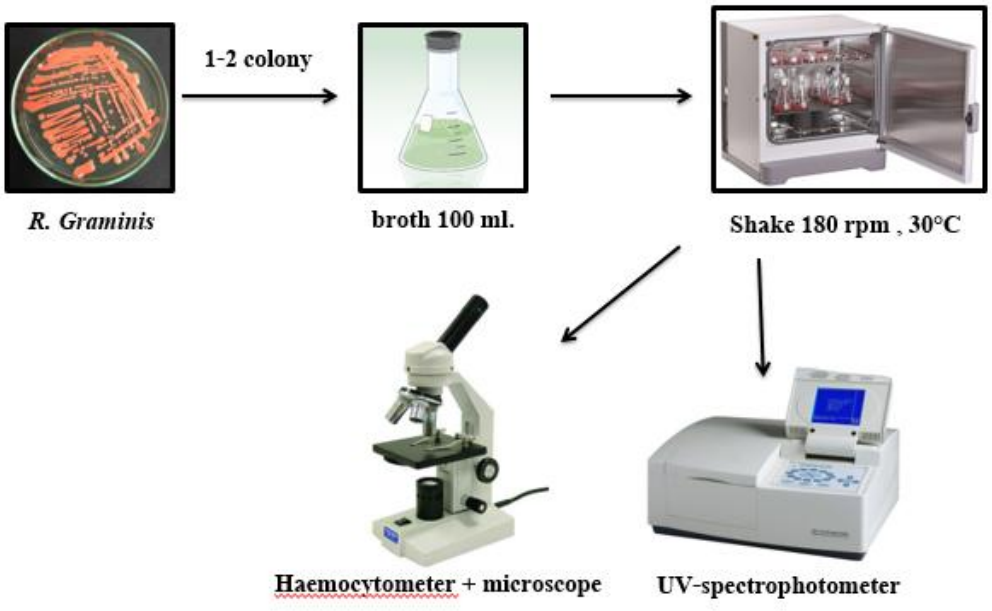

Figure 1 Schematic shows *R.graminis* cultured in the broth, cell incubation, OD measurement by UV-Vis and cell number counting

2.3. PHA Extraction

The yeast sample taken at the highest point during the exponential phase was centrifuged at 3500 rpm for 15 minutes. The pellet was washed with distilled water once before 4% of sodium hypochlorite was added and left for 1 hour at room temperature. Then the suspension was re-centrifuged at 3500 rpm for 15 minutes. Acetone and distilled water were added into the pellet containing PHA and centrifuged at the same speed for 15 minutes. The supernatant was removed and finally, chloroform was added into this pellet and dried at 65°C until chloroform was completely evaporated. The schematic of PHA extraction is shown in Figure 2. The extracted sample was examined by FTIR spectroscopy. The PHA dry weight was determined after drying at 105°C until the weight was constant. The PHA content was calculated by dividing the PHA dry weight by dry cell weight and then multiplying by 100. PHA yield was calculated by dividing the PHA dry weight by the amount of total sugar consumed.

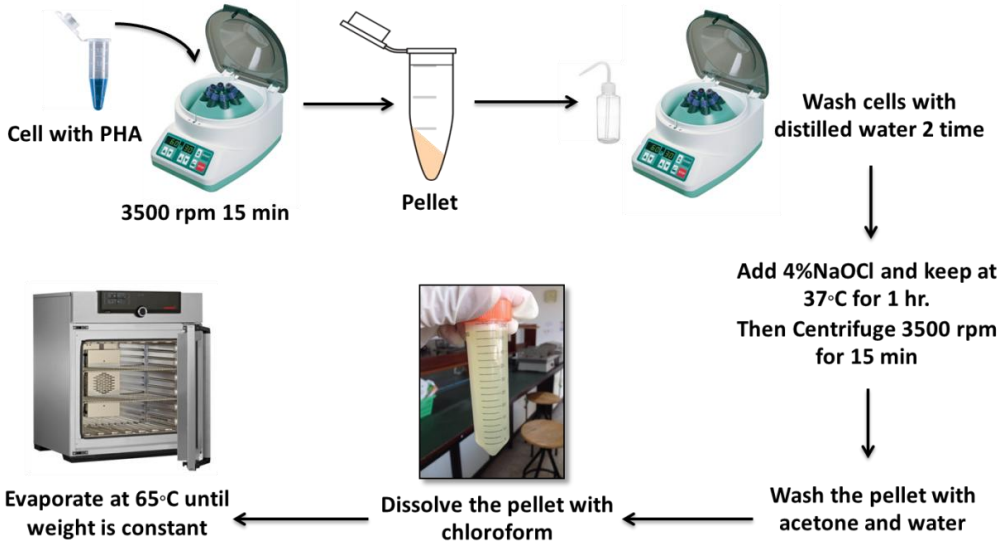

Figure 2 Schematic illustrates the method to extract PHA from yeast cells

3. RESULTS AND DISCUSSION

Optical density (OD) and cell number of yeast *Rhodotorula graminis* TISTR 5124 measured at the interval time of cells cultured under P-limited condition is displayed in Figure 3. It can be seen that during lag phase, cells adapt themselves to the growing conditions and have not divided until 45 h where it is at the beginning of log phase. Cells grew very rapidly until the end of log phase at 81 h in which cells stopped doubling and the growth became limited in stationary phase. The results of cell growth in baseline and N-limited conditions are summarized in Table 2. Principally, the yeast cell sample was taken at the end of the log phase where the maximum numbers of cell can be obtained. From Figure 3, the cell number of 3.1×10^9 cell/mL and OD of 0.95 at the end of log phase after 81 h of incubation were taken for further PHA analysis.

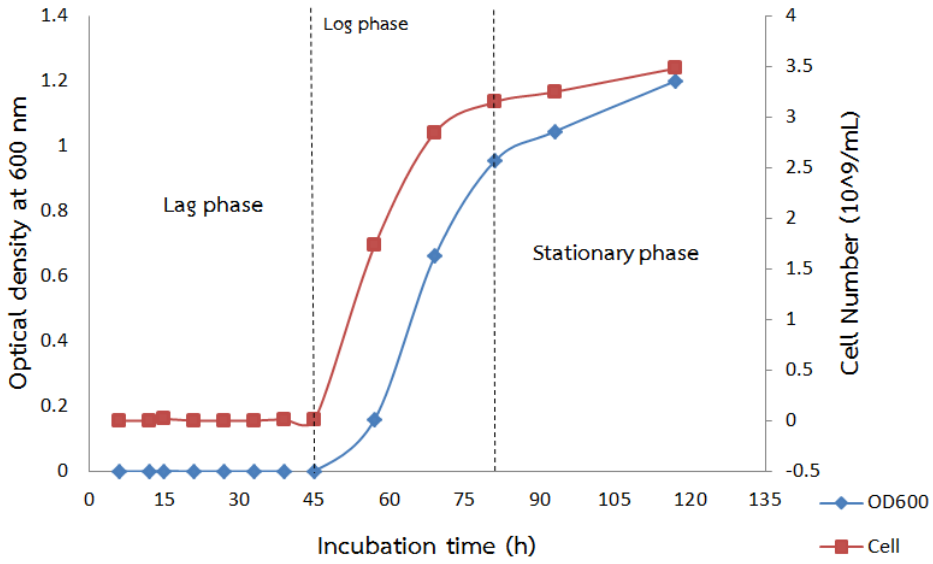

Figure 3 OD 600 nm and cell number of *Rhodotorula graminis* TISTR 5124 incubated under P-limited condition

Table 2 OD and cell number in each condition

Conditions	Incubation time (h)	OD 600 nm	Cell number (cell/mL)
Baseline	93	0.26	1.9×10^9
P-limited	81	0.95	3.1×10^9
N-limited	93	0.32	2.1×10^9

The FTIR result of PHA was extracted from *Rhodotorula graminis* TISTR 5124 cultured in P-limited condition as shown in Figure 4. The spectrum exhibited an asymmetric CH_2 of monomeric chain at a wave length of 2920.50 cm^{-1}. The stretching vibration at 2851.50 cm^{-1} was allocated to the symmetrical CH_3, suggesting a lesser indication of a crystalline polymer from a conformational disorder that occurred during the crystallization process (Liu et al., 2009). A remarkable peak of C=O bond in the carbonyl ester group at the wavelength of 1734.91 cm^{-1} was observed and this is in agreement with the FTRI spectrum of standard PHB. Absorption at 3353.91 cm^{-1} is due to the presence of hydroxyl group (OH^-) in a polymer chain (Gumel et al., 2014; Ma et al., 2009). The wavelength at 1559.85 cm^{-1} (NC=O) is assigned to a protein amid the traces remaining in the cells (Colthup et al., 1990). The stretching vibration of crystalline C-O-C at 1320-610 cm^{-1}, corresponding to the aliphatic ester is due to the polymer

crystallization of the amide band (Liu et al., 2009). The FTIR results reveal that the polymer produced by *Rhodotorula graminis* TISTR 5124 might possibly be the copolymer PHBV, when compared to the standard PHBV shown in Figure 5.

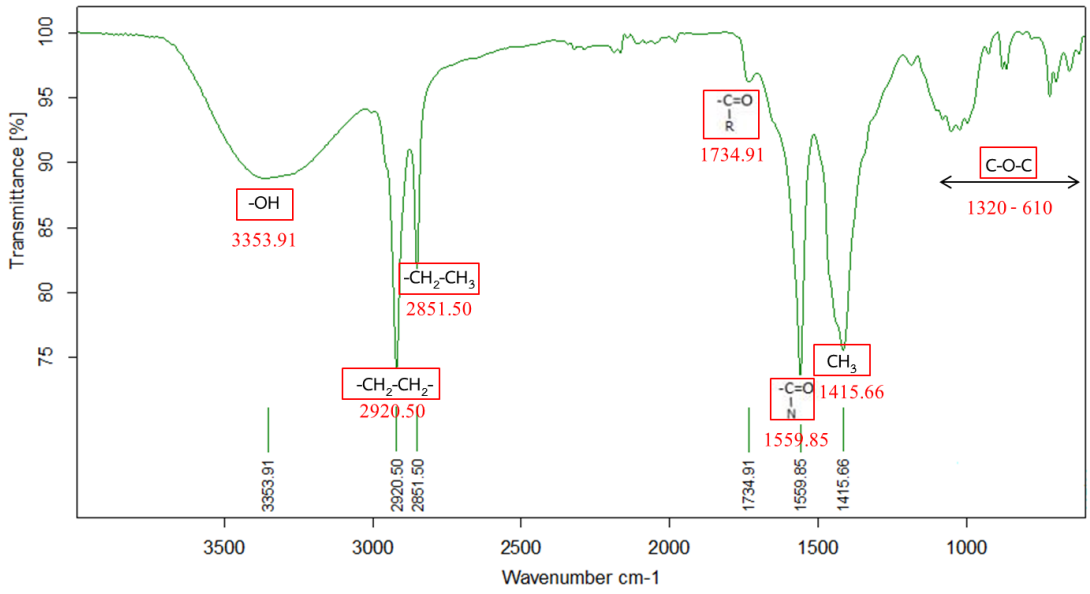

Figure 4 FT-IR spectra of PHA extracted from *Rhodotorula graminis* TISTR 5124 cultured in P-limited condition

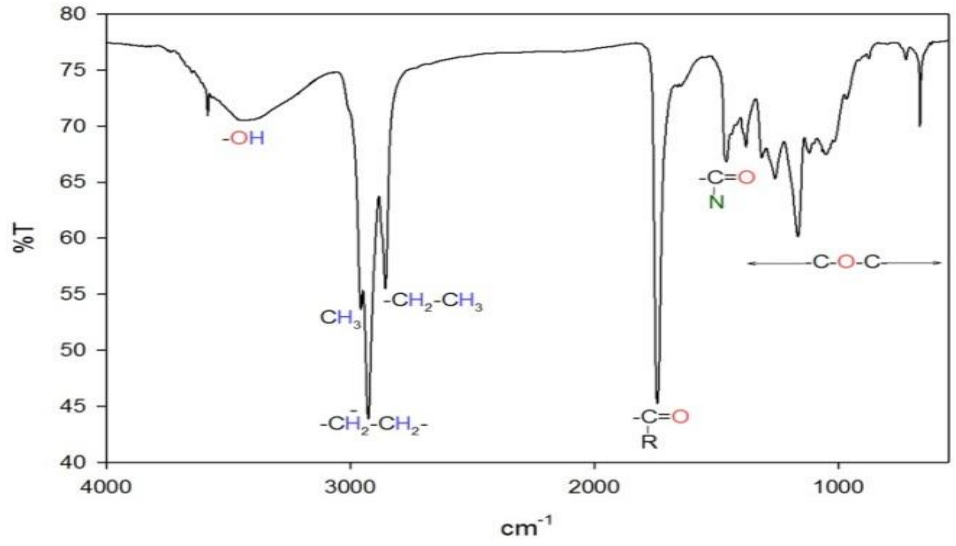

Figure 5 FT-IR spectra of copolymer PHBV extracted from *P. putida* Bet001 (Gumel et al., 2014)

The extracted polymer was also determined by ^{1}H NMR and the result is shown in Figure 6. When compared with the NMR spectra of copolymer PHBV in Figure 7, the peaks are very similar. However, there was some contamination that resulted in small peaks, thus indicating that the sample requires a better purification method. Overall, the FT-IR and NMR results can confirm that the polymer extracted from *R.graminis* TISTR 5124 is a PHA derivative or the copolymer PHBV.

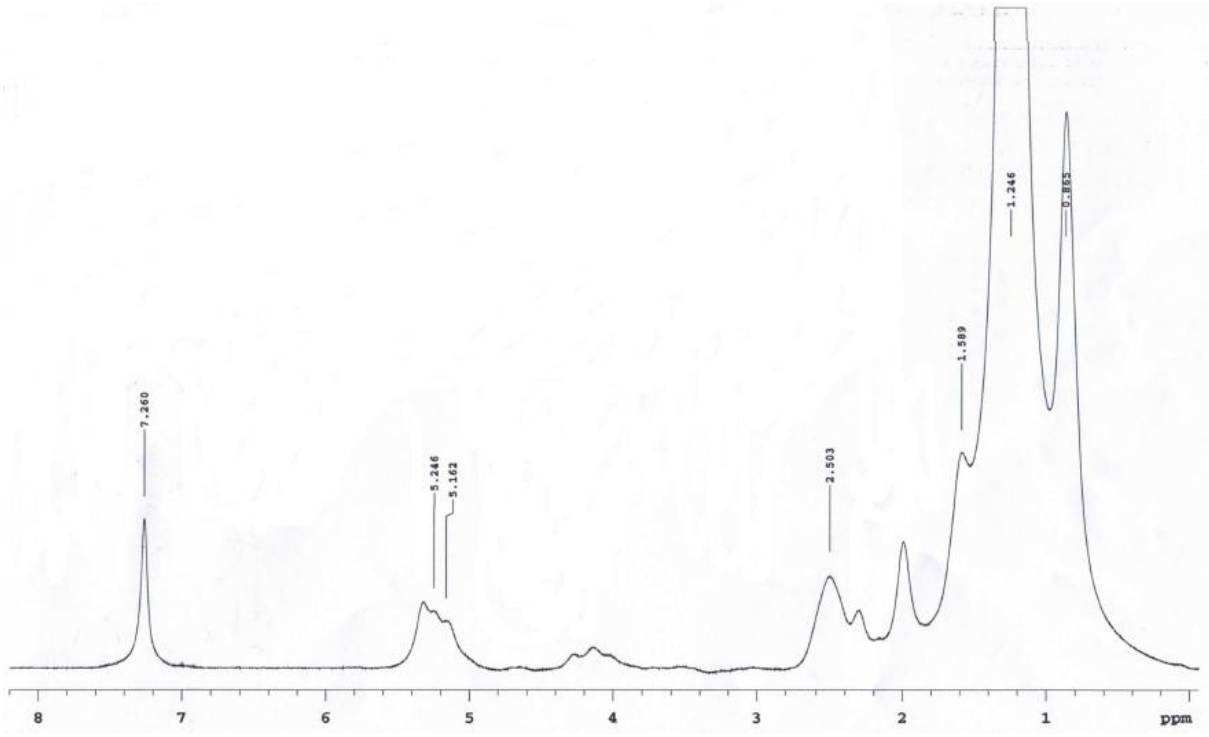

Figure 6 ^{1}H NMR spectra of extracted PHA from *R.graminis* TISTR 5124 under P-limited condition

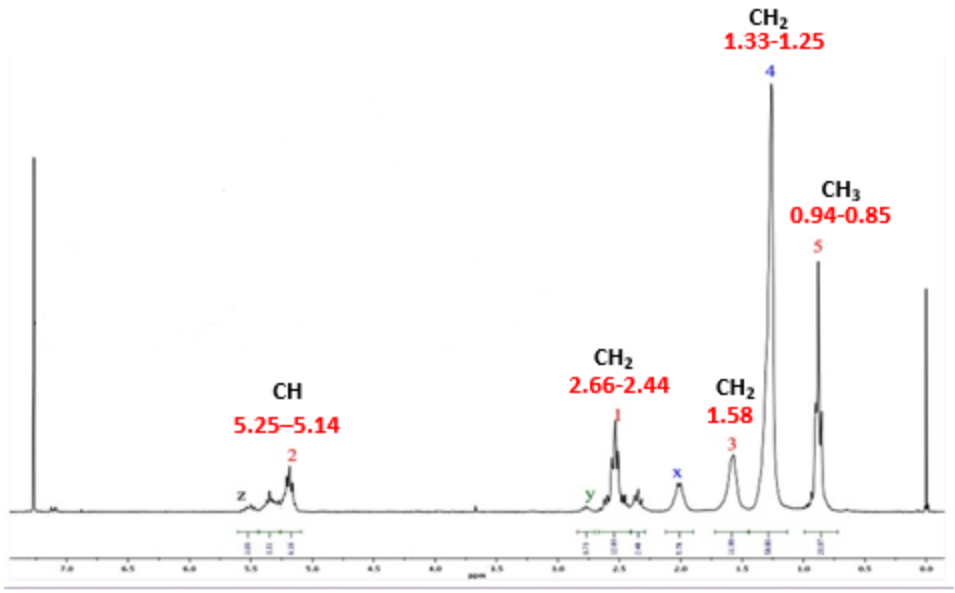

Figure 7 ^{1}H NMR spectra of copolymer PHBV extracted from *P. putida* Bet001 (Gumel et al., 2014)

The amount of glucose consumed and the PHA production in each condition is shown in Table 3. It can be seen that P-limited condition resulted in the highest PHA yield of 65.1%, where the yeast consumed a minimum glucose amount of 3.2 g/L and cell growth reached the end of log phase (81 h) more rapidly than in other conditions (93 h). This is likely due to ammonium ions in ammonium sulfate that increased the alteration of the metabolic PHA biosynthesis pathway. Nevertheless, this finding is in contrast with Poomipuk et al., (2014) who reported a N-limited condition that gave the greatest PHA content at 61.6% and a PHA yield of 0.20 g/g-total sugar

consumed in bacteria *Cupriavidus* sp. KKU38 (Poomipuk et al., 2014). This was thought to be due to the difference in nutrient demand between yeast and bacterial cells that grew before the cells were stressed and thus produced PHA.

Table 3 PHA production by *Rhodotorula graminis* TISTR 5124 in each condition

Condition	Amount of glucose consumed (g/L)	Dry cell weight (g/L)	PHA content (%)	PHA yield (g/g-total sugar consumed)
Baseline	5.4	6.2	26.1	30.0
P-limited	3.2	3.8	54.4	65.1
N-limited	5.3	6.7	34.5	43.5

4. CONCLUSION

Yeast *Rhodotorula graminis* TISTR 5124 has shown the potential to accumulate and produce a derivative of PHA, depending on the limited amounts of nutrients. Rapid growth rate and maximum PHA content of 54.4% was obtained when cells were cultured in a P-limited condition. The FT-IR and NMR spectra of extracted polymer indicate these similar stretching vibrations with the copolymer PHBV. Overall, the result is very promising that the extracted product from *Rhodotorula graminis* TISTR 5124 is a PHA derivative (copolymer PHBV). These can be applied in a scaling up process to increase the PHA yield. In conclusion, *Rhodotorula graminis* TISTR 5124 is therefore promising as a good candidate for alternatively biodegradable plastics, considering the potential to produce PHA and its derivatives.

5. ACKNOWLEDGEMENT

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