Original Article

Simultaneous Reclamation of Sago Starch Processing Effluent Water and Rhizopus oligosporus Cultivation at Different pH Conditions

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ABSTRACT

Industrial sago starch extraction from the sago palm (Metroxylon sagu) generates large volumes of wastewater, known as sago effluent that is generally discharged into nearby water bodies without proper treatment. This practice has led to severe environmental pollution that prompts the development of biotechnological treatments of sago effluent. In this study, Rhizopus oligosporus was grown in sago effluent at several initial pHs (pH 4, 5, and 6) during submerged fermentation to determine the optimum pH for high protein fungal biomass (HPFB) production while simultaneously reducing the starch content and high organic loads of sago effluent. Our results showed that the growth of R. oligosporus was the highest (3.8 g/L) when the initial pH of the sago effluent was 4. The same pH also gave the best reduction of starch, biochemical oxygen demand and chemical oxygen demand of the sago effluent following the R. oligosporus fermentations, which were 96.70%, 89.81%, and 78.30%, respectively. In addition, nitrate concentration was found to be reduced from 0.266 to 0.257 g/L, while the nitrite level dropped from 0.040 to 0.029 g/L. The present findings presented the potential of R. oligosporus for the production of HPFB as well as for treating sago effluent.

Keywords: Rhizopus oligosporus, Metroxylon sagu, sago effluent, water reclamation, high protein fungal biomass (HPFB)

INTRODUCTION

Sago starch, an essential source of starch in many parts of South East Asia, is extracted from the trunks of the sago palm (Metroxylon sagu) [1,2]. A typical sago processing mill usually processes about 1,000 sago logs daily. This practice generates approximately 7 tons of effluent that are highly rich in organic materials, chemical oxygen demand (COD), biological oxygen demand (BOD) and total suspended solids (TSS) [3,4]. According to Rashid et al. [5], the BOD, COD, and TSS in sago effluent are in the range of 910 to 1,300 mg/L, 780 to 5,130 mg/L and 19 to 20,000 mg/L, respectively. As most sago starch extraction plants are operated at small-scale, treating sago effluent loaded with high concentrations of organic materials is a challenge [4]. Conventional effluent treatment methods such as the ponding system, anaerobic digestion, mechanized treatment, and bioreactors are expensive, and a typical sago starch plant cannot afford the cost [6–9]. Therefore, the usual practice is usually to discharge the sago effluent directly into the nearby waterways, causing severe eutrophication that negatively affect the surrounding aquatic environments [2]. Without proper treatment and enforcement from the authorities on wastewater disposal, sago effluent generated by these plants would inevitably lead
to severe water pollution [2,4]. Therefore, cost-saving and efficient treatment methods need to be developed.

One possible treatment method is by subjecting sago effluent to submerged fermentation (SmF) to biologically convert the complex substrates into simpler compounds through the action of various microorganisms such as bacteria and fungi [4,10]. Filamentous fungi such as Rhizopus oligosporus, Rhizopus oryzae, and Mucor spp. are prime candidates for this kind of application [11,12]. These fungi are classified Generally Regarded As Safe food-grade fungi that are regularly used as starter cultures in the production of various traditional food [11,13]. Based on a study by Huang et al. [14], the utilization of fungi in wastewater treatment is advantageous as it requires low operating costs from the use of inexpensive raw materials, and no specific nutrient is needed for its growth. van Sambeek et al. [15] reported that R. oligosporus cultivation removed approximately 60% of organic materials, including suspended solids and several specific substances that are undesirable for recycling. Moreover, R. oligosporus tolerates low pH, and it is easy to separate the pellet biomass from the fermentation broth [16]. The fungal pellets can easily be harvested as a food-grade feed for farm animals that is rich in fat and protein. Specifically, it has a high content of the essential amino acids lysine and methionine [12,13]. Besides, this high protein fungal biomass (HPFB) is also rich in high levels of soluble protein and peptide phosphorus, chitin, and β-glucans that are known to have desirable health benefits to animals with their antioxidant and antimicrobial properties [11,12].

Although several studies have documented the cultivation of R. oligosporus in industrial wastewater, none has reported its use on sago effluent [13,15,16]. Furthermore, these studies have not explored the options of optimizing R. oligosporus growth in different pH conditions. According to Hu et al. [17], pH is one of the essential environmental parameters that directly affect the growth and formation of cellular bio-products. Generally, the effect of pH on cell growth and accumulation of cellular products vary depending on the microorganisms involved and the composition of the medium. Without proper pH control, rapid acidification could occur in the cultivation of R. oligosporus, subsequently inhibiting the production of the fungal biomass [18]. In terms of the pH of the fermentation medium, acidic conditions (pH below 4) tend to decrease the lytic activities of digestive enzymes. A slightly acidic environment is preferred in order to avoid bacterial growth during the fermentation process [18]. It is reported that the production of fungal biomass is also significantly improved with pH control. Therefore, this study aimed to investigate the potential of R. oligosporus in the production of HPFB as well as treating sago effluent via SmF at different initial pH values. The efficacy of the treatment of the sago effluent was assessed based on the reduction of several wastewater parameters such as total starch, BOD, COD, nitrate, and nitrite concentrations.

MATERIALS AND METHODS

Rhizopus oligosporus culture preparations

Rhzizopus oligosporus obtained from locally purchased tempeh was isolated and cultured on Sabouraud agar (pH 5.6) (Thermo Fisher, Hampshire, UK) at 37°C for 72 hr. Purified colonies were subcultured in Yeast Malt Broth (YMB) (pH 6.2) (HiMedia Laboratory, Mumbai, India). For long-term storage, the stock culture was aliquoted in YMB supplemented with 20% (v/v) glycerol and stored in an ultralow temperature freezer (Sanyo-MDF-U500VX-PE, Petaling Jaya, Malaysia) at -20°C [8].

Polymerase chain reaction (PCR) amplification, DNA sequencing, and species confirmation

Identification and confirmation of the R. oligosporus isolates were performed by Polymerase Chain Reaction (PCR) and DNA sequencing. The genomic DNA of the R. oligosporus isolate was extracted using DNeasy® Blood and Tissue KIT (QIAGEN, Hilden, Germany), followed by PCR amplification using a 96-well Thermal Cycler (Sensor Quest, Gottingen, Germany). The PCR reaction mixtures consisted of 2.5 µL of 10X PCR buffer, 1.0 µL of 10 mM dNTPs mix, 0.5 µL Taq DNA polymerase, 1.5 µL of 25 mM magnesium chloride (MgCl<sub>2</sub>), 1.0 µL of each 5 pmol/μl 26S-D1F (5’-GCATATCAAAAGCGGAGGAAAAG-3′), 26S-DIR (5’-GTCCCGTTTCAAGACGG-3′) primers [19], 2.0 µL of DNA template and sterile distilled water at a final volume of 25 µL. The thermocycling conditions were as follow: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation of DNA at 72°C for 1 min; with a final extension at 72°C for 10 min [19]. Upon completion of the PCR amplification, PCR products were loaded into wells of a 1.4% agarose gel in 1X TBE buffer (Fermentas Corp, Fitchburg, USA) and electrophoresed at 70 V for 2 hr. A 100 bp DNA ladder (Fermentas, Thermo Scientific, Waltham, USA) was included as molecular weight markers [19]. The gel was stained with ethidium bromide and viewed under ultraviolet light to confirm successful PCR amplification [8]. Once the presence of the amplified DNA was confirmed, the purified
amplicons were sent for sequencing. The results of the DNA sequences were then compared to the NCBI databases (www.ncbi.nlm.nih.gov) for species confirmation.

**Collection and pre-treatment of sago effluent**

Sago effluent (pH 5.6) was obtained from Herdsen Sago Mill in Pusa, Sarawak. Initially, sago hampas was separated from the sago effluent via filtration using a 710 μm mesh size filter. Suspended solids were removed from the effluent while the supernatant was collected and transferred into sterile 2 L glass bottles (SCHOTT, Duran®, Wertheim, Germany) to be used for fermentation.

**R. oligosporus fermentations with different initial pH**

The fermentation by *R. oligosporus* was conducted in modified 2-L glass bottles equipped with three silicone tubes for air inlet, air outlet, and sampling, as shown in Fig. 1. The air inlet tube was attached to an air pump (SB2800, SOBO, Guangdong, China), and the sampling tube was connected to a peristaltic pump (Masterflex Easy Load L/S 7518–00, Cole Parmer, Petaling Jaya, Malaysia). Both the air inlet and outlet tubes were connected to 0.45 μm nylon filters fitted to an air diffuser. The bioreactors were autoclaved at 121°C for 15 min prior to fermentations.

The fermentations were performed at ambient temperature in two stages: propagation and accumulation, using the same bioreactor setup, as shown in Fig. 1. YMB medium was used for the propagation stage, while sago effluents with pH adjustments were used during the accumulation stage. Once the propagation stage has reached the optimal number of days, the accumulation stage was then initiated. *R. oligosporus* was grown in YMB until the optimal day of fermentation before being transferred with the broth into pre-sterilized sago effluent for the accumulation stage. The effects of different initial pH values on *R. oligosporus* fermentations were investigated. The initial pH of the sago effluent was adjusted to 4, 5, and 6 using 1 M sulfuric acid and 1 M sodium hydroxide. The pH measurement was performed using a pH meter (Cyberscan pH 510 meter, Eutech Instruments, Bayan Lepas, Malaysia). The initial pH level of the fermentation medium was maintained by adding 0.1 M citrate buffer. The buffer for each pH was prepared to give an approximate pH, which was subsequently adjusted to the desired pH using sulfuric acid or sodium hydroxide, whenever necessary [16]. The buffers were added to 1.8 L sago effluent in 2 L glass bottles and were sterilized at 121°C for 15 min.

**Biomass analysis**

For biomass analysis, 150 ml of culture samples were collected every 24 h for six consecutive days. The samples were centrifuged at 5,000 rpm for 10 min at 4°C, and the supernatant was recovered for further analyses. The harvested pellets of biomass were dried in an oven at 80°C for 48 h and were quantified gravimetrically. The growth of *R. oligosporus* is expressed as the increase in the dry cell biomass as a function of time (day) on a volumetric basis.

**Phenol-sulfuric total carbohydrate analysis**

Phenol-sulfuric total carbohydrate analysis was performed by dispensing 1.0 ml of supernatant into a test tube, followed by 4 ml of distilled water. The solution was then mixed well by vortexing. Next, 0.2 ml of the sample solution was transferred into a new test tube, and 0.2 ml of 5% (w/v) phenol solution was added. The contents of the test tubes were mixed again by vortexing before adding 1.0 ml of 98% (v/v) sulfuric acid. After vortexing, 5.6 ml of distilled water was added into each tube, and the mixture was then vortexed. One milliliter of the mixture was then transferred into a quartz cuvette (Nerbe Plus, Winsen, Germany), and the absorbance of the suspension was measured at 490 nm using a spectrophotometer (SP-880, Metertech Inc., Taipei, Taiwan). The equivalent sugar concentration (g/L) was determined based on a standard curve of glucose generated prior to the assay.
Nitrate and nitrite analysis

Nitrate and nitrite concentrations in the supernatant then were analyzed using the API Nitrate and Nitrite Test Kit (API, McLean, USA) according to the manufacturer instructions, with slight modifications. The absorbance of the supernatant was determined at 528 nm both nitrate and nitrite concentrations, respectively. Analyses were performed by initially construction standard curves using 0 to 200 g/L nitrate and 0 to 10 g/L nitrite standard solutions. To perform nitrate and nitrite analyses on the samples, 3 ml of aliquots were transferred to a fresh centrifuge tube, and centrifuged at 13,500 rpm for 3 min. The supernatant was then tested using the same test kits in triplicates.

Biological oxygen demand

The BOD values were determined using APHA Method 5210 B [20]. Dilution water was prepared before the analysis by adding 1 ml of phosphate buffer, magnesium sulfate, calcium chloride, ferric chloride, and sodium sulfate into a 1 L Schott bottle, and the final volume was made to 1 L with distilled water. The dilution water was then aerated overnight with a Zone Z-80 Air pump (KW Zone, Johor Bahru, Malaysia). The BOD of the supernatant was measured using a dissolved oxygen (DO) meter (Milwaukee MW600, Rocky Mount, USA). One ml of sample was mixed with approximately 299 ml of dilution water in the BOD bottle, and the initial DO level of the water sample was recorded. The bottles were then incubated in the dark for five days. After the incubation, the DO of the samples was measured, and the BOD was calculated using equation (1).

\[
\text{BOD}_5, \text{mg/L} = \frac{(D_1 - D_2) - S \times V_s}{P}
\]  

(1)

where: 
\(D_1\) = DO of diluted sample immediately after preparation, mg/L 
\(D_2\) = DO of diluted sample after 5 d incubation at 20°C, mg/L 
\(S\) = oxygen uptake of seed, DO/ml seed suspension added per bottle 
\((6 \text{ d}) \ (S = 0 \text{ if samples are not seeded})\) 
\(V_s\) = volume of seed in the respective test bottle, ml 
\(P\) = decimal volumetric fraction of sample used; \(1/P\) dilution factor

Chemical oxygen demand

The COD value was determined by using APHA Method 5220 C [20]. Firstly, 2.5 ml of diluted supernatant was pipetted into a vial containing 1.5 ml of potassium dichromate (\(K_2Cr_2O_7\)) digestion solution and 3.5 ml concentrated AgH-SO\(_4\) acid solutions before being heated at 150°C in a COD reactor for 2 h to oxidize the entire organic load completely. The COD for a blank sample was also included. After the solution cooled down, it was then transferred to a conical flask, and the excess dichromate was titrated with Ferrous (II) Ammonium Sulfate (FAS) solution. A Ferroin indicator was used, and titration was carried out until the color of the solution changed from greenish blue to brownish red. The volume of FAS needed to achieve the end-point of the titration was recorded, and the COD was calculated according to equation (2).

\[
\text{COD, mg O}_2/\text{L} = \frac{(A-B) \times M \times 8,000}{\text{ml sample}}
\]  

(2)

where: 
\(A\) = ml FAS used for blank 
\(B\) = ml FAS used for sample 
\(M\) = molarity of FAS 
8,000 = milliequivalent weight of oxygen \(
\times 1,000 \text{ ml/L}
\)

Statistical analysis

The statistical analyses were carried out using SPSS software (SPSS Statistics Version 21, SPSS Inc., New York, USA). Tukey’s test was performed for the comparison of means via Analysis of Variance (ANOVA) at 95% confidence interval. The data were expressed as mean ± standard deviation (SD) from three independent parallel experiments. The error bars were determined based on the standard deviation of the mean values.

RESULTS AND DISCUSSION

Isolation and identification of \(R.\) oligosporus

\(R.\) oligosporus was isolated from locally purchased tempeh. PCR amplification performed on the DNA isolated from presumptive isolates generated a DNA fragment of approximately 650 bp in length (Fig. 2), which is within the size range for the 26S-D1F and 26S-D2R locus [19]. From the DNA sequencing results and BLAST analyses [21], a 100% identity match to known the sequences of \(R.\) oligosporus was established.

Propagation and accumulation stage of submerged fermentation of \(R.\) oligosporus in sago effluent

The growth of \(R.\) oligosporus in sago effluent was quantified in order to determine the feasibility of the fungi to fer-
ment the sago effluent (94.91 ± 0.09% moisture, 2.23 ± 0.86% free starch and 2.86 ± 0.06% solids), thus resulting in the production of HPFB and reduction of organic loads. In this study, a two-stage continuous culture system was developed for *R. oligosporus* fermentations. The initial propagation stage was conducted to identify the optimal fermentation period for *R. oligosporus* growth in YMB medium. During the fermentations, constant aeration was provided to the bioreactors in order to provide sufficient oxygen for cell growth. According to van Leeuwen *et al.* [21], insufficient oxygen supply may result in a decrease in fungal growth, as most fungi are obligate aerobes, and molecular oxygen in the substrate is required for their growth. In the second stage, the effect of different initial pH of sago effluent on *R. oligosporus* fermentation was investigated. The growth of *R. oligosporus* was monitored daily by measuring the dry biomass. The highest dry biomass of *R. oligosporus* obtained in the propagation stage was 3.36 g/L on day 6. Henceforth, the fermentation period of *R. oligosporus* in YMB was standardized to 6 days.

The growth and biomass production of fungi vary when cultured under different cultivation conditions [23,24]. These conditions are generally affected by several parameters, including pH [18,25]. The biomass production by *R. oligosporus* in sago effluent under different initial pH is presented in Fig. 3. The control experiment used sago effluent with no modification of the initial pH (pH 5.6). In all fermentations, the growth patterns of the fungi were found to be similar regardless of the initial pH of the sago effluent. *R. oligosporus* cultured in sago effluent exhibited three main phases of growth: lag, exponential, and stationary phases, as observed in most microorganisms [26]. The length of each phase was different, depending on the availability of nutrients in the fermentation medium [27]. During the initial propagation stage, the culture concentrations were relatively smaller as the fungal cells were in preparation for cell division [28]. *R. oligosporus* cultures grown at the initial pH of 4, and the control adapted rapidly to new fermentation conditions, while those in pH 5 and 6 responded more slowly, resulting in longer lag phases. The length of the lag phase is typically influenced by several factors, including the physiological conditions of cells, the initial inoculum size, and the culture conditions of both the original and new growth medium [29,30]. The duration of the lag phase is inversely dependent on the initial inoculum size, where a small inoculum size results in more extended periods of lag phase.

The biomass production by *R. oligosporus* was the highest on Day 6 for all pH conditions tested. Except for fermentations with the initial pH of 6, all other fermentations showed progressive increments in biomass during the exponential phase. As illustrated in Fig. 3, the highest growth of *R. oligosporus* was achieved in fermentations with the initial pH of 4, with the biomass production of 3.80 g/L attained on Day 6. Meanwhile, the highest biomass concentration achieved in the control experiment and fermentations with the initial pH of 5 and 6 were 2.07, 1.51, and 1.25 g/L, respectively. These indicate that the *R. oligosporus* biomass production was affected by pH values. pH readings taken throughout
the fermentation stage revealed no significant changes in all experiments. As shown in this study, pH 4 was found to be the optimal pH for the growth of *R. oligosporus* in sago effluent. This implies that an acidic environment can contribute to better production of *R. oligosporus* biomass. This is in agreement with the findings by Jin *et al.* [16], who reported pH 4 as the optimal pH for the growth of *R. oligosporus* in other types of wastewater. Meanwhile, Nagel *et al.* [18] reported that the growth rate of *R. oligosporus* did not significantly vary between pH 4 and 6. In another study, the optimal growth condition for most fungi is acidic, with a pH of lower than 5 [22]. This is because the acidic environment can prevent the growth of most bacteria, thus providing a less competitive environment suitable for fungal growth [18].

**Phenol sulfuric analyses**

Figure 4 shows the residual starch concentrations during the fermentation of *R. oligosporus* in sago effluent under different initial pH, as determined via phenol sulfuric analysis. Overall, a gradual decrease in starch concentration was observed throughout the fermentation period. In terms of the total percentage of starch reduction, fermentations with the initial pH of 4 showed the highest reduction of 96.70%, followed by the control experiment, and fermentations with the initial pH of 5 and 6, with their reduction of 87.09%, 84.41%, and 82.04%, respectively. These results are in agreement with a study by Jin *et al.* [16] that demonstrated the capability of *R. oligosporus* to convert more than 95% starchy compounds in starch processing water. As the fermentation progress, the nutrients present in the sago effluent, such as starch, were consumed by *R. oligosporus* in order for them to grow and multiply. These observations are supported by van Leeuwen *et al.* [22] that verified the degradation and absorption of polysaccharides from the growth media by *R. oligosporus*. *R. oligosporus* can perform this task as it produces glucoamylase that enhances the conversion of starch to glucose [16].

**Nitrate and nitrite analysis**

Nitrate is commonly found in all types of water sources, including drinking water and wastewater [31]. In this study, the nitrate analysis was performed to quantify the nitrate ions present in the fermentation media of different initial pH. Figure 5 shows the reduction of nitrate and nitrite in all *R. oligosporus* fermentations. In terms of the total reduction of nitrate ions throughout the fermentation period, fermentations performed with the initial pH of 4 showed the highest reduction of 3.38%, followed by the control and fermentations with the initial pH of 5 and 6, which were 2.89%, 2.51%, and 2.21%, respectively (Fig. 5A). The reduction of nitrate levels could be contributed by the consumption by *R. oligosporus* for its growth. According to van Leeuwen *et al.* [22], nitrate is one of the nitrogen sources, and most fungi can utilize inorganic nitrogen as they are capable of decomposing protein.

The concentration of nitrite ions throughout the *R. oligosporus* fermentations in sago effluent was also analyzed. As shown in Fig. 5B, nitrite concentrations for all experiments decreased gradually from Day 0 to Day 6. The highest nitrite reduction (27.73%) was shown in the fermentations performed with the initial pH of 4. Meanwhile, fermentations with the initial pH of 6 showed the lowest reduction percentage of 11.18%. The data obtained from the current analysis showed that the concentration of nitrite was lower compared to nitrate. The same observations are in accordance with the study by Praus [32], which reported that the concentration of nitrite was usually slightly lower than nitrate, as nitrite is an unstable form of nitrogen and is easily oxidized to nitrate. Although nitrate and nitrite concentrations in carbohydrate-rich sago effluent are low, and these parameters seem negligible, this study still demonstrates the potential their removal by *R. oligosporus* cells.

![Fig. 4 Starch reduction during SmF of *R. oligosporus* in sago effluent at different initial pH. Data are shown as mean ± SD. Error bars correspond to the standard deviations of triplicates.](image_url)
Biological oxygen demand

Figure 6A illustrates the BOD profiles of all R. oligosporus fermentations under different initial pH. The BOD level of the sago effluent with pH modification was found to be higher than the control experiment. This is because the addition of buffers, sulfuric acid, and sodium hydroxide to adjust and maintain the pH has indirectly added foreign substances to the system, hence increasing the BOD value. High initial BOD values of the sago effluent indicate the presence of organic compounds, indicating the low concentration of dissolved oxygen in the sago effluent. In all fermentations of R. oligosporus in this study, the BOD levels throughout the fermentation period. This observation can be correlated with the rapid decrease of DO during the exponential growth of the fungal biomass [16]. The highest reduction of BOD in this study (89.81%) was observed in fermentations with an initial pH of 4, whereas the lowest reduction (78.70%) was observed in fermentations with an initial pH of 6. The remarkable reduction of BOD during the R. oligosporus fermentations in sago effluent, as shown in this study, suggests that the wastewater reclamation in sago effluent was successful, although it was not a full recovery.

Chemical oxygen demand

The COD of the sago effluent during the R. oligosporus fermentations were found to decrease in all experiments conducted. The highest reduction of 78.30% was observed in fermentations, with an initial pH of 4. The reduction percentage for the control experiment and fermentations with the initial pH of 5 and 6 were 70.08%, 65.30%, and 50.85%, respectively. These values also suggest notable COD reduction when R. oligosporus was cultured in sago effluent. COD measures the pollution potential from organic matter in water bodies. The COD analysis uses a strong oxidant, potassium dichromate, in a concentrated sulfuric acid solution and is refluxed at 150°C to oxidize organic carbon to CO₂ and H₂O. The COD test uses a chemical that oxidizes both organic and inorganic substances in a sample, which results in a higher value than the BOD for the same sample, as only organic compounds are consumed during BOD testing [33]. Theoretically, the COD for fermentation media with pH adjustment would be slightly higher than those without adjustment. The addition of buffer, acid, and base added foreign substances to the system, resulting in a higher COD value. Final COD values were still relatively high, suggesting that longer periods of cultivation coupled with other forms of tertiary treatments may be introduced as supplementary treatment processes. Another possible explanation to this observation is the carry-over of compounds from the initial propagation broth at the first stage of fermentation.

Table 1 shows the summary of the maximum biomass production, starch consumption, and parameters related to the wastewater treatment as obtained on Day 6 in all R. oligosporus fermentations performed using sago effluent with
different initial pH values. In summary, the results showed that the initial pH of 4 used to cultivate *R. oligosporus* in sago effluent during SmF outperformed the other pH values in all parameters tested, such as HPFB production and the reduction of starch, nitrate, nitrite, BOD, and COD at notable statistic differences.

**CONCLUSIONS**

This study has demonstrated the potential of *R. oligosporus* for the production of HPFB as well as the treatment of sago effluent. The results showed that the initial pH of the sago effluent that was used as a medium for *R. oligosporus* fermentations has a direct effect on the *R. oligosporus* biomass produced as well as the reduction of starch, nitrate, nitrite, BOD, and COD. This work provides useful insights into the development of sustainable and low-cost approaches for treating sago wastewater besides creating a fascinating route for the production of value-added products such as *R. oligosporus* HPFB.

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