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Biodegradation of natural rubber latex film added with *Metroxylan Sagu* pith form by *Bacillus cereus* ATCC 14579

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ABSTRACT

Aims: This study is focused on the potential of *Bacillus cereus* ATCC 14579 to degrade natural rubber (NR) latex film and NR latex film added with *Metroxylan sagu* pith waste (NR/TSPW latex film).
Methodology and results: *Bacillus cereus* is proved capable to utilise rubber as main source of carbon and energy. The biodegradation studies were analyzed by growth profile, weight loss, protein content and tensile strength test. Fourier Transform Infrared Spectroscopy (FTIR) test was applied to confirm the biodegradation process. In shake cultures, OD of culture increased by 19.2% from the initial inoculum after 14th cultivation days with NR/TSPW latex film. An increase in protein content up to 0.037 mg/g with 12.377% weight loss of film was obtained after biodegradation. Tensile test result shows tensile strength and elongation break are decreased by 10.203%.
Conclusions, significance and impact of the study: The demand of rubber products is increasing time to time. Due to the high consumable and disposable of rubber latex products, hence remain inert to degradation and leading to their accumulation in the environment. An attempt to combine Natural Rubber (NR) latex system with other degrading materials have been made to facilitate biodegradation process. Thus, *Bacillus cereus* ATCC 14579 has potency to provide a biotechnological solution to the waste rubber disposal problem.

Keywords: Biodegradation, *Metroxylan sagu* waste, rubber latex

INTRODUCTION

Rubber product takes a long period of time to degrade naturally and causes major environmental problems in the municipal landfills. Plus, the reuse and recycling of rubber latex products are inefficient, not green ways and required sophisticated equipment to solve with the environmental problems addressed (Chia *et al*., 2014). Thus, some ideas have been made to incorporate starch-containing waste as bio-filler during compounding of natural rubber (NR) to form biopolymers (Singhal *et al*., 2008; Awg-Adeni *et al*., 2010). The previous research studies have proven that the introduction of sago starch can improve the biodegradation properties of latex products (Chew *et al*., 1993; Awg-Adeni *et al*., 2012; Uthumporn *et al*., 2014). However, food crisis issue raised and treated *Metroxylan sagu* pith waste (TSPW) is used to replace sago starch in the natural rubber compounding system.

Metroxylan sagu is the scientific name of sago palm. It comes from 'metra', means pith and 'xylon', means xylem. Meanwhile, sago pith is the inner part of the sago palm trunk. The rasped pith of the sago palm is extracted to harvest sago starch. During sago starch extraction process, about 60 to 80 wt% of sago starch can be harvested (Afiq and Azura, 2013a; 2013b). Therefore, NR latex is added with TSPW to speed up the biodegradation process of films (TSPW/NR).

In biodegradation process, the rubber degrading bacteria consume NR as the only carbon and energy sources for their growth. To date, previous studies have shown most of microorganism only degrade latex samples with no filler (Jendrossek *et al*., 1997; Chandra and Rustgi., 1998; Linos *et al*., 2000; Bode *et al*., 2001; Rose and Steinbüchel, 2005; Cherian and Jayachandran., 2009). This study focused on identification of NR degrading bacteria and its biodegradation process on both NR latex and NR/TSPW latex film.

MATERIALS AND METHODS

Natural Rubber (NR) Latex film samples

The NR films samples were prepared earlier. Each sample was buried in compost soil for 12th week of biodegradation period, respectively. In the meantime,

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TSPW/NR latex films were prepared for the further studies.

Identification of bacteria

One g of soil buried NR latex films was mixed to 100 mL of mineral salts medium in flask. The flask was agitated on 100 rpm at 30 °C for 3 days. Serial dilutions are conducted to decrease the concentration of bacteria in the sample. Subsequently, 100 µL of each dilution were disperse on latex overlay agar. The agar plates were put in the incubator at 30 °C for 7 days. The colonies with dissimilar morphology were selected and transferred on a fresh nutrient agar (Sigma-Aldrich) until pure colony is obtained.

According to Braaz *et al.* (2004), latex overlay agar was prepared using mineral salts medium (MSM) by the overlay methods. The composition of MSM was based on the ingredients performed by Heisey and Papadatos (1995): 2.0 g/L $NaNO_3$, 0.5 g/L $MgSO_4$, 0.5 g/L KCl, 0.01 g/L $Fe_2(SO_4)_3 \cdot H_2O$, 0.14 g/L KH_2PO_4, 1.2 g/L K_2HPO4 and added with 0.02 g/L of yeast extract. Meanwhile, the addition of 1.5% of bacteriological agar powder was mixed with MSM for agar preparation. During the agar preparation, 0.6% of purified natural rubber (NR) latex was added to mineral salts medium.

Starch hydrolysis was studied by observing the disappearance of starch on the plate, which followed the composition: 3 g/L beef extract, 5 g/L peptone, 2 g/L soluble starch and 15 g/L bacteriological agar under pH 7.2. The bacterial culture was spot on the centre of starch agar plates and incubated at 37 °C for 24 to 48 h. The plate was fulfilled with Gram's iodine for 10 min (Abiola and Oyetayo, 2016).

Biodegradation test

The washed films were cut into small pieces and the exact initial weight was recorded. Approximately 1 g of films were dissolved in 100 mL of acetone for 12 h and allowed to dry, respectively. The treated films were autoclaved to maintain a sterilize condition before the introduced them into culture medium. Thereafter 10% of inoculum (*Bacillus cereus* ATCC 14579) was added into 150 mL of mineral salts medium. Approximately, 300 mg of treated films was added into the culture. The culture was incubated on 150 rpm for 14 days at 30 °C. The study was carried out with three replicates for each NR and TSPW/NR latex films. Control set was performed with minerals salts medium added with treated films, respectively without inoculums. The optical density (OD) of culture was measured at 600 nm. The films were separated from culture before centrifugation, washed and dried until constant weight was achieved. The final weight was recorded. The control sets were harvested at 14th days of cultivation.

Molecular identification

Molecular analysis was carried out by Centre for Chemical Biology (CCB), Penang, Malaysia. GF-1 Bacteria DNA Extraction Kit was used to obtain pure DNA sample. Further, the purity of DNA was determined by Nanodrop 2000 Spectrophotometer. The 16S rRNA gene was augmented by PCR using universal primers 16S-27F and 16S-1492R. PCR was conducted under 94 °C (3 min), 30 cycles of 94 °C (30 sec), 55 °C (30 sec) and 72 °C (1.4 min) and a cycle of final extension at 72 °C (5 min) conditions. The sequences were identified using BLAST analysis from National Centre for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Protein determination

Protein contents of *B. cereus* grown at different NR latex film were determined according to the method of Bradford (Ernst and Zor, 2010). The harvested cultivation media to be tested were prepared after filtering the culture via syringe filters 0.45 µm pore size, respectively. The 10 µL of sample was mixed with 200 µL Bradford reagent (5× dilution, Sigma-Aldrich) in a 96 well microtiter plate. The solution was left at room temperature in dark condition for 10 min. The absorbance was measured at 595 nm by using microplate reader (Halo MPR-96, Dynamica Ltd). Four samples were used and the average results were calculated.

Weight loss

Upon collection after 14th cultivation days, the films were rinsed and dried at 30 °C to a constant weight. The weight loss of the samples with time was applied to examine the degradation rate of the samples by following Equation 1,

$$\text{Weight loss (\%)} = [(W_i - W_d)/W_i] \times 100 \quad (1)$$

where W_i is the initial dry weight of the sample and W_d is the dry weight of sample after biodegradation

Mechanical properties test

Tensile strength test was performed using Instron® 3366 Machine (Norwood, MA, US) as followed to ASTM D 412-06 at room temperature. The dumbbell shapes of control NR and TSPW/NR latex films were prepared. The crosshead speed was fixed at 500 mm/min. The average readings of four samples were recorded.

FTIR spectroscopy test

FTIR spectrometer (Perkin-Elmer Model Series 2) was carried out to study the changes in chemical functions on the surface of latex films. The peak height represent the IR spectrum, which is correspond to absorbance. IR spectra were gained using attenuated reflectance. The scaning range of 4000-400 cm^{-1}, with resolution of 4 cm^{-1} were used to collect the FTIR spectra.

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RESULTS AND DISCUSSION

Isolation of bacteria

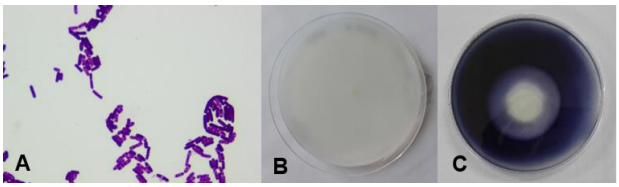

Figure 1: Macroscopic and microscopic characteristics of *B. cereus*. Colonies of *B. cereus* under light microscope 1000× (A), on MSM agar (B) and starch agar (C).

There are various bacteria can hydrolyse starch, however, a capability to hydrolysed rubber is limited to a few genera (Rose and Steinbüchel, 2005). Therefore, the research was conducted first to select the rubber-degrading bacteria rather than to examine their capability to degrade modified NR. The modified NR used is TSPW/NR latex films.

The isolate *Bacillus cereus* ATCC 14579 was chosen because of its fastest growth on latex overlay agar. *B. cereus* is Gram-positive bacteria (Figure 1A) with 2-7 mm in diameter. These non-pathogenic bacteria were found to have asymmetrical, with undulate, crenate and fimbriate borders, and matt or granular surfaces as described in the (Bergey *et al*., 2000). However, the drawback of latex overlay agar is not all rubber-degrading bacteria able to form clear zone area on such plates. It's because only little amount of isoprene available on the plate that are not compatible with some bacteria growth system (Rose and Steinbüchel, 2005).

Although *B. cereus* is non-clear zone former on latex overlay agar (Figure 1B), it showed positive reaction to Gram's iodine on starch agar (Figure 1C). The formation of clear zone around *B. cereus* indicates the starch is hydrolysed. A blue zone shows the starch hydrolysis is not occurred. During the growing phase, the starch molecules are not too large to diffuse across the cell membrane. Thus, some species of bacteria, including *B. cereus* secrete exoenzyme amylase to degrade starch into subunit that can be utilized by them (Abiola and Oyetayo, 2016).

Biodegradation studies

The biodegradation study was conducted to further verify the rubber-degrading ability of *B. cereus*. Figure 2A showed the growth of *B. cereus* in the cultivation medium. The OD of culture for TSPW/NR latex film was extremely highest. The OD of culture NR latex film increased by 10.5% from 0.227 on day 0 to 0.332 on 14th days. Meanwhile, the OD of the culture increased by 19.2% in TSPW/NR latex films. The increment of OD from 0.204 on day 0 to 0.396 on 14th days indicated the growth of *B. cereus* faster than NR latex film.

Protein content measurement also been used as evidences of biodegradation of NR and TSPW/NR latex films by *B. cereus (*Table 1). After 14th cultivation, an increase in protein up to 0.037 mg/g after incubated TSPW/NR latex film with *B. cereus*. In contrast, an increase 0.030 mg/g in the protein content was found for NR latex film.

Figure 2B showed the weight loss of the NR and TSPW/NR latex films during 14 days of cultivation in mineral salts medium by *B. cereus*. It is clearly showed *B. cereus* can accelerate the biodegradation process of films with compare to control film. Studies show that, a weight loss of 7.799% was achieved for NR latex films and a 12.377% weight loss for TSPW/NR latex films under these state.

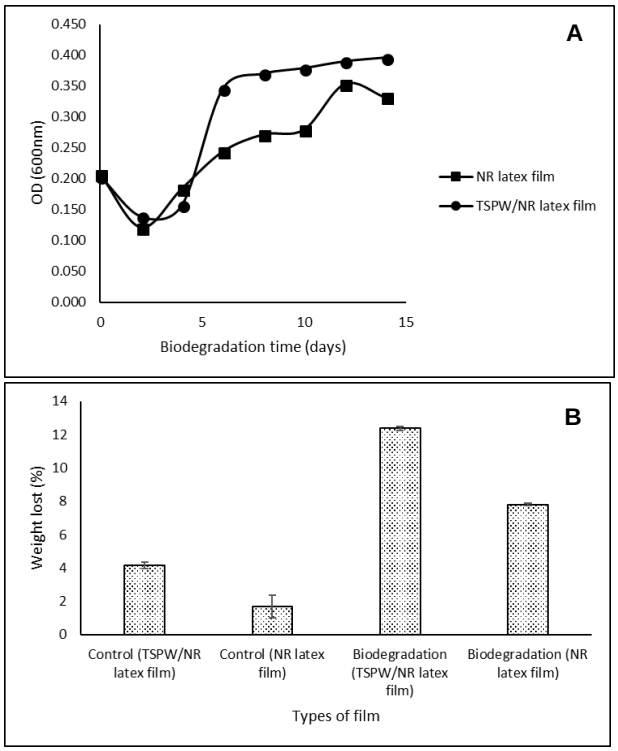

Figure 2: The growth of *B. cereus* (A) and the weight loss of films after 14 days of biodegradation periods (B).

Table 1: Protein content after 14 days biodegradation periods.

Types of film	Protein content (mg/g)
Control (TSPW/NR latex film)	0.027
Control (NR latex film)	0.023
Biodegradation (TSPW/NR latex film)	0.037
Biodegradation (NR latex film)	0.030

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Tensile strength analysis

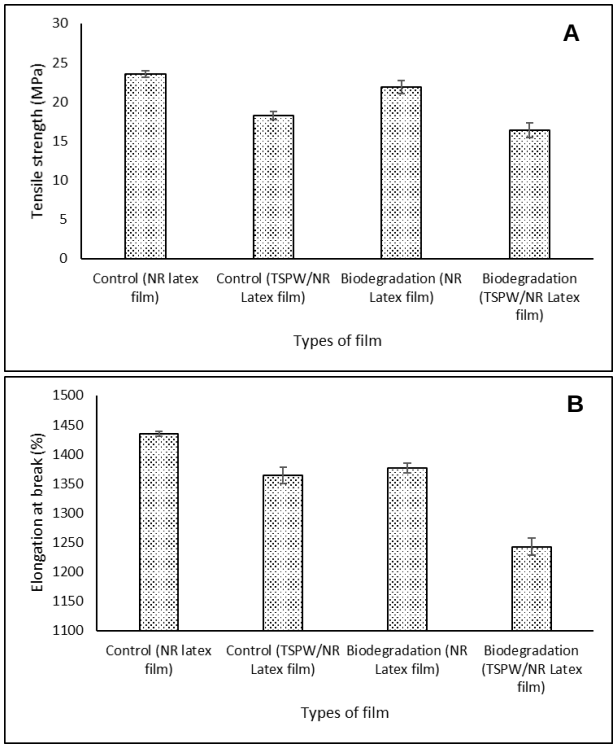

Figure 3: Effect of biodegradation process on films; tensile strength (A) and elongation at break (B).

For control films, the introduction of TSPW in NR latex films decreased in tensile strength (Figure 3A) due to poor formation of sulphur crosslinking in the rubber particles. The TSPW/NR latex films subjected the lowest tensile strength and elongation at break (Figure 3B) after biodegradation periods. This indicated *B. cereus* more favoured to hydrolyse glycosidic linkage using starch hydrolysis enzyme compared to polyisoprene chain (Rose and Steinbüchel, 2005). As the breakage of these bonds, the films could not resist the stress applied hence decreased the tensile strength of the films.

FTIR spectroscopy Test

Examination of a graph (Figure 4i and 4ii) showed FTIR profile for different types of films. The films, which were cultivated by *B. cereus* were resulted for FTIR studies peaks were observed at the wavelength between 550-4000 cm^{-1} as described at Table 2.

Oxidation process cleave polyisoprene chain at double bond, which between two carbon atoms. During the bond scission, the formation of aldehyde and ketone groups can be recognized as the chain is cleaved by homolysis process, with epoxide group as intermediates (Linos *et al*., 2000; Roy *et al*., 2006; Isa *et al*., 2007).

Table 2: Major peaks in the FTIR spectra for NR and TSPW/NR latex film

Wavenumbers, cm^{-1}	Description
3200 – 3600	OH stretching (Chandra and Rustgi., 1998)
2800 – 2960	Methyl and methane group streching
1736	CO stretching vibration means the formation of aldehyde and ketones group (Rose and Steinbuchel., 2005)
1638	OH bending of the absorbed water
1440	Lignin of TSPW
1376	In-the plane CH bending
1010 – 1120	Unsymmetrical C-O-C and C-O bond of primary alcohol showed. Methyl and methane group vibration. The epoxide group observed.
780 – 920	Double bond vibration Methane group vibration in starch (Isa *et al*., 2007)

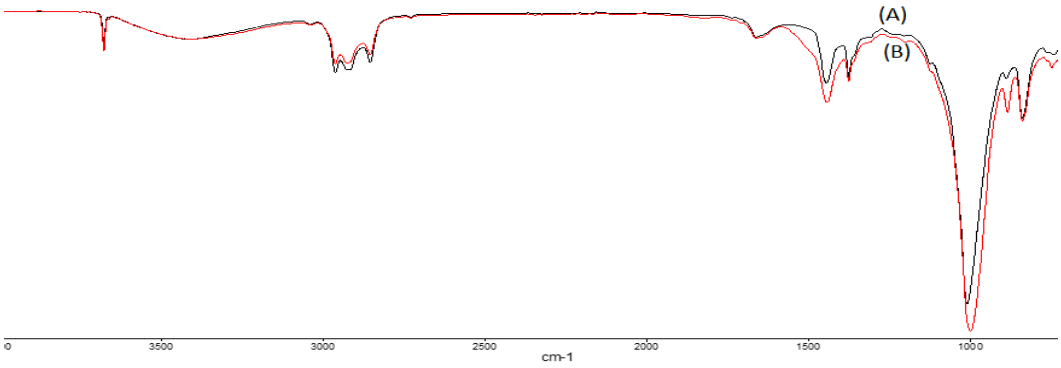

Figure 4(i): FTIR profile for different types of film with Control (NR latex film) (A), Control (TSPW/NR latex film) (B).

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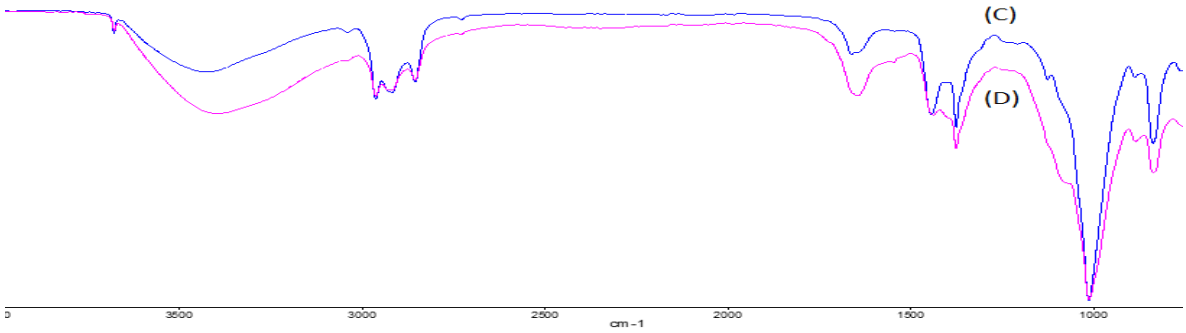

Figure 4(ii): FTIR profile for different types of film with Biodegradation (NR latex film) (C), Biodegradation (TSPW/NR latex film) (D).

CONCLUSION

Bacillus cereus ATCC 14579 isolated from NR latex film is a non-clear zone NR degrading bacterium but showed positive result to the starch utilization. It is identified by colony morphology and 16S rRNA analysis. Most of the studies on the biodegradability have been clearly visualize by its growth profile, protein content and weight loss. Analysis of FTIR showed the decreased in double bonds and the formation of carbonyl groups (ketone and aldehyde group). The spectra proved the polyisoprene chain and sago starch were being used as carbon sources during biodegradation by *B. cereus*. Although the introduction of TSPW decreased films tensile strength in NR latex film, it will help to promote the biodegradation process. Hence, the introduction of TSPW in rubber products can degrade by *B. cereus* and further, the solid waste disposal management improved.

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REFERENCES

Abiola, C. and Oyetayo, V. O. (2016). Isolation and biochemical characterization of microorganisms associated with the fermentation of Kersting's groundnut (*Macrotyloma geocarpum*). *Research Journal of Microbiology* **11, 47-55.**

Afiq, M. M. and Azura, A. R. (2013a). Effect of sago starch loadings on soil decomposition of Natural Rubber Latex (NRL) composite films mechanical properties. *International Biodeterioration and Biodegradation* **85, 139-149.**

Afiq, M. M. and Azura, A. R. (2013b). Utilization of starch to accelerate the growth of degrading microorganisms on the surface of natural rubber latex films. *Journal of Chemistry and Chemical Engineering* **7, 137-144.**

Awg-Adeni, D. S., Aziz, S. A., Bujang, K. and Hassan, M. A. (2010). Bioconversion of sago residue into value added products. *African Journal of Biotechnology* **9, 2016-2021.**

Awg-Adeni, D. S., Aziz, S. A. Bujang, K. and Hassan, M. A. (2012). Recovery of glucose from residual starch of sago hampas for bioethanol production. *BioMed Research International* **2013, 1-8.**

Bergey, D. H., Buchanan, R. E., Gibbons, N. E. and American Society for Microbiology. (2000). Bergey's manual of determinative bacteriology (9th Edn.). Williams and Wilkins. Baltimore.

Bode, H. B., Kerkhoff, K. and Jendrossek, D. (2001). Bacterial degradation of natural and synthetic rubber. *Biomacromolecules* **2, 295-303.**

Braaz, R., Fischer P. and Jendrossek, D. (2004). Novel type of heme-dependent oxygenase catalyzes oxidative cleavage of rubber (poly-cis-1,4-isoprene). *Applied and Environmental Microbiology* **70(12), 7388-7395.**

Chandra, R. and Rustgi, R. (1998). Biodegradable polymers. *Progress in Polymer Science* **23, 1273-1335.**

Cherian, E. and Jayachandran, K. (2009). Microbial degradation of natural rubber latex by a novel species of *Bacillus* sp. SBS25 isolated from soil. *International Journal of Environmental Research* **3(4), 599-604.**

Chew, T. Y. and Shim, Y. L. (1993). Sago processing waste in Kuala Lumpur: Waste management in Malaysia - Current status and prospects for bioremediation. Ministry of Science, Technology and the Environment. **pp.159-167.**

Chia, K. H., Nanthini, J., Thottathil, G. P., Najimudin, N., Haris, M. R. H. M. and Sudesh, K. (2014). Identification of new rubber-degrading bacterial strains from aged latex. *Polymer Degradation and Stability* **109, 354-361.**

Ernst, O. and Zor, T. (2010). Linearization of the Bradford protein assay. *Journal of Visualized Experiments* **38, 1918.**

Heisey, R. M. and Papadatos, S. (1995). Isolation of microorganisms able to metabolize purified natural rubber. *Applied and Environmental Microbiology* **61, 3092-3097.**

Isa, S. Z., Yahya, R., Hassan, A. and Tahir, M. (2007). The influence of temperature and reaction time in the

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

degradation of natural rubber latex. *The Malaysian Journal of Analytical Sciences* **11, 42-47.**

Jendrossek, D., Tomasi, G. and Kroppenstedt, R. M. (1997). Bacterial degradation of natural rubber: A privilege of actinomycetes? *FEMS Microbiology Letters* **150, 179-188.**

Linos, A., Reichelt, R., Keller, V. and Steinbüchel, A. (2000). A gram-negative bacterium identified as *Pseudomonas aeruginosa* AL98, is a potent degrader of NR and synthetic cis-1,4-polyisoprene. *FEMS Microbiology Letters* **182, 155-161.**

Rose, K. and Steinbüchel, A. (2005). Biodegradation of natural rubber and related compounds; recent insights into hardly understood catabolic capability of microorganisms. *Applied and Environmental Microbiology* **71(6), 2803-2812.**

Roy, R. V. Das, M. Banerjee, R. Bhowmick, A. K. (2006). Comparative studies on rubber biodegradation through solid-state and submerged fermentation. *Process Biochemistry* **41(1), 181-186.**

Singhal, R. S. Kennedy, J. F. Gopalakrishnan, S. M. Kaczmarek A. Knill C. J. and Akmar, P. F. (2008). Industrial production, processing and utilization of sago palm-derived products. *Journal of Carbohydrate Polymer* **72, 1-20.**

Uthumporn, U. Wahidah, N. and Karim, A. A. (2014). Physicochemical properties of starch from sago (*Metroxylon Sagu*) palm grown in mineral soil at different growth stages. *Materials Science and Engineering* **62(1), 012026.**

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