

## CAPTURING ANTHOCYANIN IMMOBILIZATION ON RICE THROUGH THE ULTRA-HIGH RESOLUTION ELECTRON LENSES

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**Abstract.** Coating parboiled rice grains (PRG) with pigment-immobilized nanometer thickness cells abbreviated "pinacell" offered rice grains with more uniform *Clitoria ternatea* (flower) pigment distribution. The resultant blue pinacell, predominantly of delphinidin anthocyanin, was then examined for its penetration into the rice grain using an ultra-high resolution Field Emission Scanning Electron Microscopy (FESEM) and soft peeling from polystyrene surface. Pinacell-polystyrene surface tension occurring at 40°C facilitated delamination, signaling another prospectus commercialization candidate and the possibility of a zero-waste process. Analysis of the coated grain cross-section via XHR FESEM shows pigment penetration to the core of the grain plausibly delivered first, by the nanofibrils slipping into the inter-granular gaps, IGS. Next, the anthocyanin adhered to the PRG core, driven by its biochemical affinity for starch and proteinaceous structures of the PRG. The thermal canopy effects arising from the muffling web of pinacell and the thermo-pockets forming at PRG surface IGS during the 40°C drying, coupled with the antimicrobial characteristic of *Clitoria ternatea* pigment rendered profound preservation measures. The identified coating and penetration mechanisms account for better grain longevity, color uniformity, and an enhanced shelf-life placing the coated parboiled rice closer to market readiness.

**Keywords:** Nano-enabled Food Technology, Biomass, Functional cells, FESEM, anthocyanin.

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## Introduction

Food industries have been relentlessly innovating better preservation and processing technologies [1]. The nano-enabled technology fronts aim to produce "high-performance" food technology materials in devices used in process control, innovative packaging and storage [2], rheology, or direct add-ons for extra dose aesthetic, longevity, and sensorial improvements. The manifold benefits could be functionalized with antioxidants, color, aroma, mouthfeel, and emotional health benefits to the food generally taken for satiation.

Rice, the food crop contributing to 2% of Malaysia's GDP (Gross Domestic Product), is one food that can be prepared in fanciful ways. *Nasi lemak*, *nasi hujan panas*, *nasi Mendy*, *nasi ayam*, and *nasi ulam* are rice-based servings with added nutritional values due to herbal flavor add-ons like ginger, clove, coconut milk or coconut oil, saffron, and health-enhancing herbs. Some of the nutrient-enriched food require a long preparation time. Similarly, if the rice preparation time is taken from the time the herbs are purchased, delivered, or plucked from the tree, the process can be extremely long.

*Kerabu* rice or *nasi kerabu* is no new treat in Southeast Asia. It is famous for its light taste, soft-blue energy dish balanced with satiating and nutritious proteinaceous add-ons like fish, chicken, red meat, fibrous cabbages, fancy paprika, coconut, and super-healers like *ulam raja*. The blue color stems from the squeezed flowers of *Clitoria ternatea* (butterfly pea). Hidden within the scrumptious meal is the medicinal property known to strengthen and recover bodily vitality [3-6].

The violet-hued blue pigment is not only nutritious but the healthy door to avoiding artificial coloring associated with the known cytotoxic and carcinogenic effects. In 2007, the Consumers Association of Penang (CAP) lauded the ban of artificial food coloring due to its ill effects on children's hyperactivity and various allergies. Besides rice, *Clitoria ternatea* flower pigment is also a famous coloring agent used in bakeries, glazing, beverages, cosmetics, and therapeutics. Thus far, microencapsulation using maltodextrin [4] inspired by the earlier attempt on *B. Vulgaris* anthocyanins [5] was reported equivalently successful for *C. ternatea* petals (CTP). Similarly, the use of Arabic gum and gelatin were among the studied encapsulation materials. Adorned with such attractive features as enhanced readiness and rapid preparation time, pigment-coated rice is another reasonable offer to *Nasi Kerabu* fans to prepare the serving with ease despite a hectic schedule. Analogous to instant rice, pre-coated rice grains can potentially be one of the must-have kitchen inventories at fast-food restaurants and busy homes based on consumers' informed choice of the health benefits. The stigma harnessed by processed food and the cytotoxicity of non-plant food colorants such as Brilliant Blue FCF [4] can gradually be resolved by laying a spectrum of alternatives. CTP violet-blue pigment is an excellent selling point to those aware of the long-term health threat from consuming artificial colorants.

Drying is the essential process employed for the storage or commercial use of *C. ternatea* petals. While dry-blower and greenhouse are available techniques for preserving CTP, sun-drying is a natural preservation measure in preparing CTP for the market. Besides degrading the less photostable components, the open-air approach entices a host of airborne contaminants, including specks of dirt from the flying organism, exposing consumers to an unknown health risk. The dried petals also require refrigeration to achieve a six-month shelf life, not without the risk of rewetting due to the hygroscopic nature of the agricultural structure. Aware of hygienic needs, consumers may choose to wash the dried petals before squeezing the pigment. During washing, an inevitable amount of pigment leached from the

biomass due to biodegradation. Among the lost commodity of *C. ternatea* is the water-soluble blue pigment. Indeed, the wastage of therapeutics like delphinidin anthocyanin [6] can be prevented by minimizing the end-stage processing and developing a packaging design that accommodates the biomass's photostability. Today, the anthocyanin-rich, low-cost biomass is in demand for a broadened innovative application. While its use in Dye-sensitized Solar Cells (DSSC) [7] is the hallmark for the renewable energy sector, anthocyanin-based biosensors [8] will even be valued in the textile and smart apparel industry due to their unique, sophisticated chemistry [9]. Efficient management of the dye sources has now become critical.

For the intended pigment-coated *Kerabu* rice market, this study analyzed the outcome of coating the *C. ternatea* pigment on the parboiled rice. The reason for choosing parboiled rice was its reputable health claim attributed to the 90% predominance of type-three resistant starch with 8% protein and a negligible portion of lipids [10]. The constituents favorably support the human digestive system [11-13] by effectively absorbing calcium and iron [13], improving gut flora and colon health. Deliberation is also placed on the nano-enabling effects of TRX-cells<sup>TM</sup> - the nano-meter thickness cells delaminated from natural fibers to fit the overall *Kerabu* rice production process into a zero-waste management framework.

## Materials and Methods

*Clitoria ternatea* pigment was coated on the parboiled rice with and without the pre-characterized TRX-cells<sup>TM</sup>. The suspension containing the butterfly pea pigment and TRX-cells<sup>TM</sup> is hereon denoted "pinacell". Details of the experiment are described in the proceeding paragraphs.

**Clitoria Extracts.** From the planting area in the north part of Malaysia (Latitude: 5 26' 00" Longitude: 100 32' 00'), the flowers of *Clitoria ternatea* were plucked, and the petals were weighed and oven-dried for 12 hours at 40°C. The dried petals were then ground using a heavy-duty grinder and weighed once again for yield estimation. The ground sample was then sealed in a polyethylene bag and stored at -4°C. The pigment extraction was performed by soaking the sample in ethanol at a 1:1 plant biomass-to-solvent ratio and subject to an ultrasound-assisted pre-optimized extraction procedure. The process involved three-level factors for the Central Composite Design (CCD) Response Surface Methodology (RSM) experiment with 20 portions of 5 g ground petals. The parameters were ethanol (25%, 50%, 75%), time (20, 30, 40 minutes) and temperature (30°C, 40°C, 50°C). The solvent was then removed by a rotary evaporator and stored at -4°C. All chemicals were purchased from GIBCO (Waltham, USA) or Sigma-Aldrich (St. Louis, USA).

**Parboiled Rice Grains.** The high-in-demand parboiled rice grains were purchased from the Malaysian Agricultural Research and Development Institute (MARDI), Seberang Perai. The rice milling process involved soaking, partial boiling in the husk at 65-85°C [14], steaming, and intensive drying, giving rise to enhanced nutritional profile, slightly different texture, and more resistance to rice beetles. As a result of the unique milling, parboiled rice is high-fiber, and the low-glycemic index offers slow digestibility, preventing a spike in blood sugar. Amongst rice grains, parboiled rice grains are health-boosting rice with the resistant type III starch (RSIII) critical for a healthy gut.

**Pigment Immobilisation Agent.** TRX-cells<sup>TM</sup> was obtained from the delamination of the oil palm fruit-bunch microfibrils under the conditions recommended in the literature [15]. The nanometer thickness laminae were suspended in the distilled water and examined with High-Resolution Transmission Electron Microscope, HRTEM 200 kV, TECNAI G2 20 S-TWIN model to confirm its presence. The TRX-cells<sup>TM</sup> were also analyzed to confirm the dimension of the fibrils in the nanolaminae. LEO SUPRA 50 VP Carl Zeiss Scanning Electron Micrograph at the School of Biological Sciences, Universiti Sains Malaysia, was accessed for the essential routine check. Samples smeared on glass slides were mounted on the stub with double-sided electrically conducting carbon tapes and then gold-coated using Polaron Equipment Limited model E500 with a voltage of 1.2 kV and 20 Pa for 10 minutes.

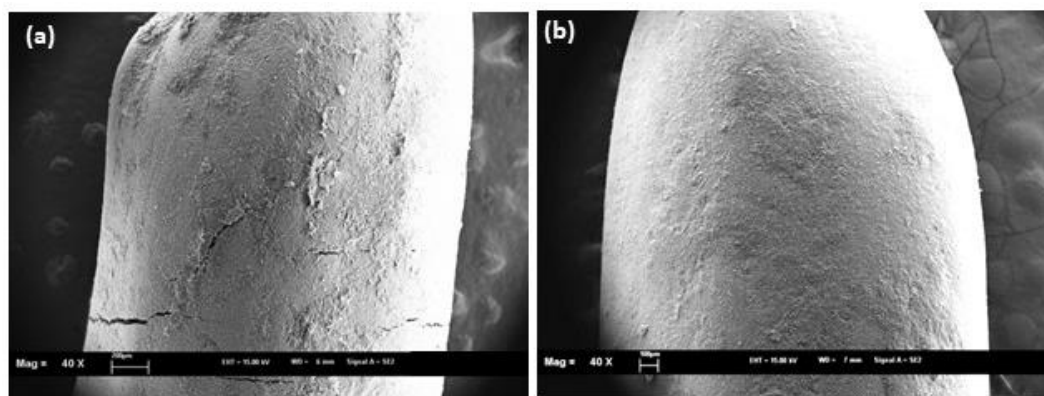
**Pinacell - Pigment Immobilisation Nano Cell Suspension.** Two concentrations of TRX-cells<sup>TM</sup> suspension were prepared, and 2.5 ml of these were mixed with the *Clitoria ternatea* pigment extract at a 1:1 volume ratio. Five grams of parboiled rice grains (PRG) placed in a stainless-steel fine-mesh cage and submerged in the pinacell suspension, swirled, and dried at 40°C for 8 hours. The selected temperature ensured the preservation of the grains and pigment therapeutic activity [16]. The coated PRG were packed in 5 g portions for treatment and analyses. All samples were monitored for signs of molding.

**Microscopic Analysis.** The spent pinacell liquor, grains, and control experiments were kept for analysis. Screening analysis was performed using a portable image analyzer to determine the various outcome of pinacell coating. The pigment immobilization profile was examined by the Extreme High-Resolution Field Emission Scanning Electron Microscope (XHR-FESEM FEI Varios 460L) at the Science and Engineering Research Center (SERC), Engineering Branch Campus, Universiti Sains Malaysia. XHR FESEM's powerful morphological contrasting effects were acquired via high-resolution electron landing energy in the 0.5 keV - 10 keV range for stable imaging and excellent contrast of the non-conductive sample [17]. Various modes of detectors were attempted to acquire the desired contrast. The whole and dissected rice grains were placed on the sample holder. Dried pinacell flakes were also examined by analyzing the smear forming on the polystyrene dish. Evidence of flaking and delamination (peeling) occurring at the polystyrene-pinacell interface were tracked with the XHR FESEM.

## Results and Discussion

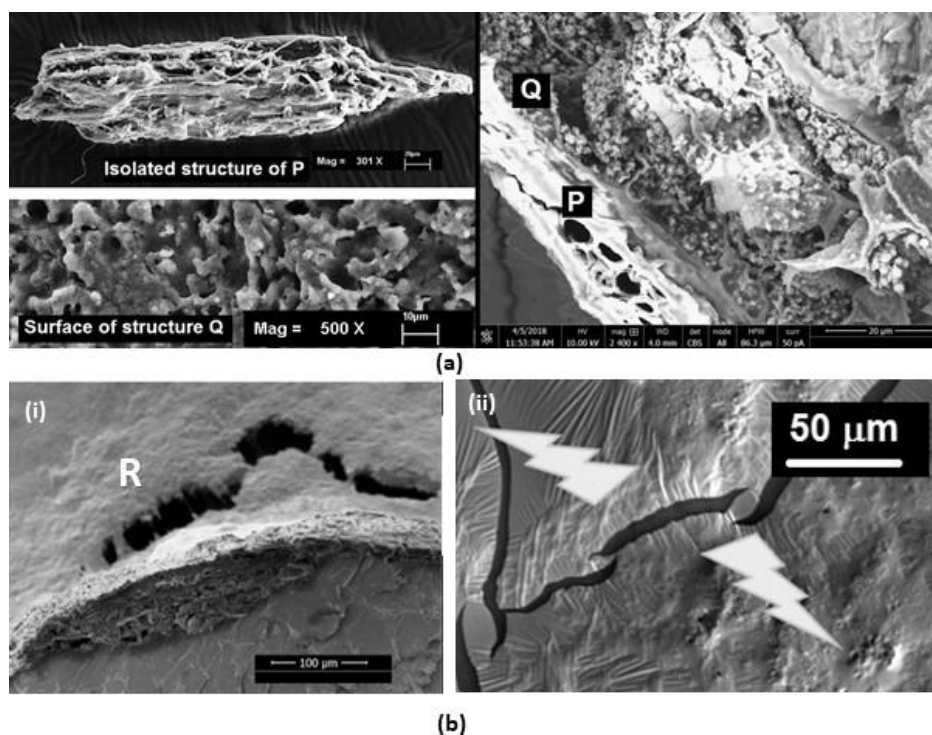
The surface, the pinacell-grain boundary, and grain cross-section of the pinacell-coated parboiled rice were examined through the electromagnetic lenses of SEM and XHR FESEM to examine the nature of the pinacell-rice interface and the anthocyanin penetration profile.

**Thermal Effects from Voids and Nano-blanket.** Besides the visible improvement in color uniformity that ruled out the reported issue with freeze-drying [18], the outcome of coating parboiled rice grains (PRG) is shown in Figure 1.



**Figure 1. Pigmented parboiled grains (a) with pinacell compared to (b) parboiled grain without coating.**

Scanning Electron Microscopy revealed evidence of surface micro-cracks (Figure 1a) attributable to the thermo-blanketing effect arising from the TRX-cell™ nanolaminiae and the micro-spaces between the insoluble flakes (P, Figure 2a) in the extracted pigment.



**Figure 2. Pinacell differing interfaces (a) close-up look at the cross-section of pigment-coated rice and (b) three-quarter view of the pinacell-rice interface (left) in the vicinity of crack and evidence of pinacell-plastic surface tension induced delamination (arrows on b(ii)).**

The micro-granular structure (Q, Figure 2a) forming the grain surface topography also created the inter-granular spaces (IGS) that acted as micro-scale thermo-pockets. The IGS thermo-pockets added to the thermo-blanketing effect that trapped heat during the drying process the pigmented grains underwent, analogous to the heat-trapping effect from (the 52% fibrous [19]) almond skin that often overcooked the core of the almond. The effect favorably explained the higher shelf life of the pinacell-coated rice grain. It was observed that without pinacell, the pigment-coated grains were more susceptible to microbial attack attributable to

the residual moisture. The findings suggest the need for an effective heat impregnation after the aqueous dip-coating process. The observation also marks the adequacy of 40°C heating for 8 hours as the measure for pinacell-coated grain preservation – the temperature that falls within the range recommended in the literature [16].

Field Emission Scanning Electron Microscopy of the rice surface (Figure 2) and cross-section shows the pigmented layer of the parboiled grains' starchy porous surface. FESEM depicts a significant contrast between rice grain's starchy surface and the fibrous pinacell coating due to the differences in the atomic characteristics of the two material phases.

The granules on the grain surface acted as a defect point, imposing enough surface tension to crack the pigment layer (Figure 2). Areas that dried at a higher rate were likely to be the escape path for internal vapors from the rice core. Analysis of the crack area reveals a fibrous bottom layer, plausibly arising from the 13.5% microfiber in *C. ternatea* extract (Table 1). Compared to the nanometers scale fibers in the TRX-cells™, the presence of oversized (60 µm length and 500 nm diameter, Figure 3a) fibers originating from *C. ternatea* petals created the initiation peeling point. Besides surface tension, the bulging sites gathered heat and dried relatively faster than the more uniform layers. While favorable for dry-peeling of pinacell, the cluttering of microfibrils in pinacell signals the need for a vigorous stirring of the pinacell suspension during the grain coating process to uniformly disperse and break the chunks into smaller pieces.

The loose granular structure (Q, Figure 2a) rendered the soft adhesion, which enhanced the peeling tendency (R, Figure 2b) of the pinacell coated rice grains. Flaking of pinacell is a minor issue as the cooking process would redeposit the color on the cooked rice. In the presence of TRX-cells™, the rice starch-fibrous pigment interface was somewhat compatible, serving simultaneously as glue to the starchy surface granules. The challenge of distinguishing between the organic component of the rice and the organic layer of TRX-cells™ coincides with the previously reported challenge [2, 20]. The delineation between these interfaces was even more challenging due to the fibrous and heterogeneous composition of the pigment (Table 1).

**Table 1. Partial Analysis of *Clitoria ternatea* Pigment**

Component	Percentage
Active Constituents	Tarexerol <sup>[19]</sup>   Steroids <sup>[21]</sup>   Triterpenoid <sup>[21]</sup>   Saponins <sup>[21]</sup>   Resins <sup>[21]</sup>   Tanins <sup>[21]</sup>   Starch <sup>[21]</sup>   Ash <sup>[22]</sup>
Fiber Portion and Diameter	2.1 <sup>[22]</sup> -13.5% (10 µm)

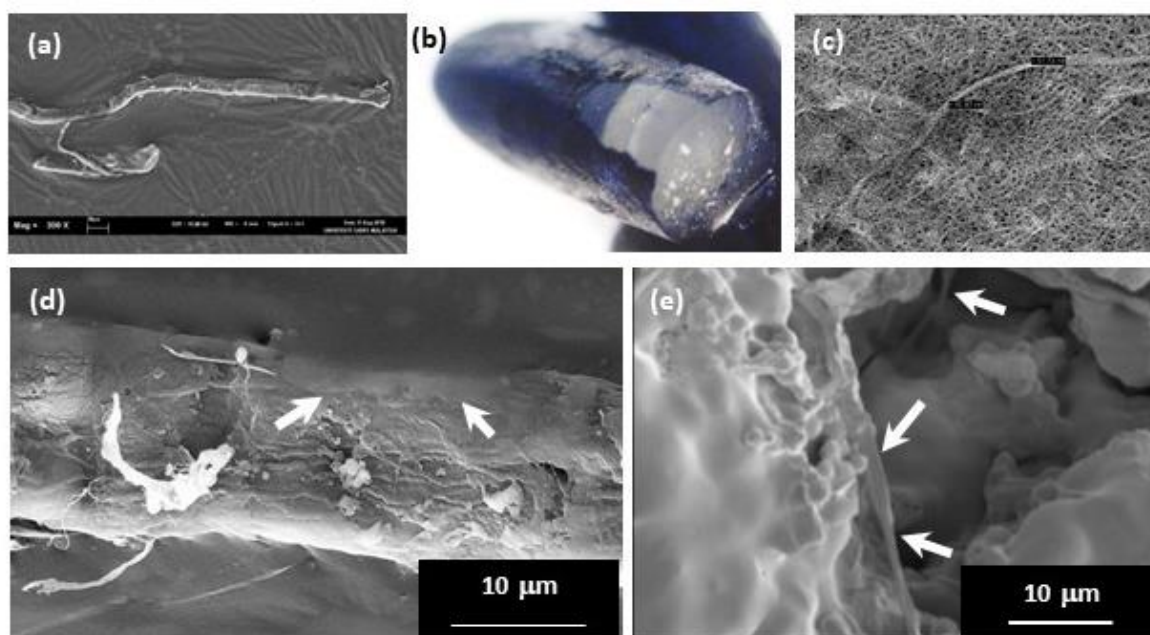
**Pigment Penetration.** The minuscule fibrils dangling on the web of TRX-cells™ also make it possible to slip into the micro inter-granular spaces (IGS) of the rice grain. The phenomenon is ascertained by the relative sizes (Table 2) of the fibrous mass in the extracts (5-20 µm diameters fibers and 1 µm diameter fibrils – Figure 3) to the size of micro-cracks which were 5 µm at maximum (Table 2).

**Table 2. Pigment Penetration Profile and Microcracks**

	Pigment Penetration			
	Depth (µm)		Penetration Pattern	
Condition	+TRX-cells <sup>TM</sup>	-TRX-cells <sup>TM</sup>	+TRX-cells <sup>TM</sup> creep-in   cross-through	-TRX-cells <sup>TM</sup> creep-in   cross-through
Delphinidin	53-70-100	9-13	✓ ✓	
	Micro-cracks Dimension		Delphinidin Pigment Fusion	
Length (µm)	150	50	10 – 150 µm	10 µm
Width (µm)	Variable	5		

+TRX-cells<sup>TM</sup> ≡ with TRX-cells<sup>TM</sup> | -TRX-cells<sup>TM</sup> ≡ without TRX-cells<sup>TM</sup>

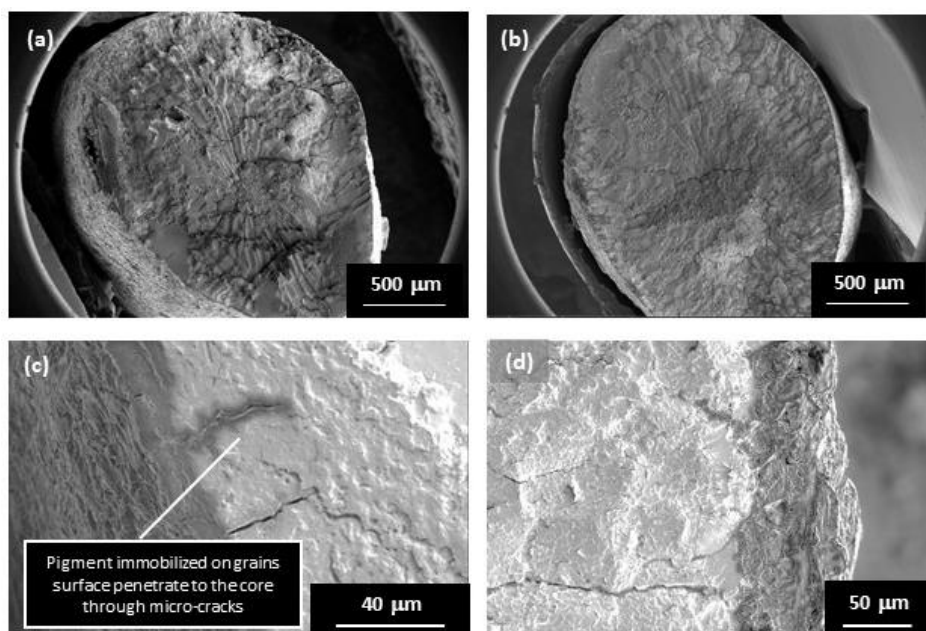
Fibrils of the TRX-cells<sup>TM</sup>, on the other hand, lie in the 90-100 nm (Figure 3c) diameter range, allowing slipping into the IGS together with the immobilized pigment to the core of the grain. As a result, delphinidin immobilized on TRX-cells<sup>TM</sup> could penetrate deeper into the core of the parboiled rice grains (Table 2). These measurements were made on the rice grains coated with and without pinacell (Figure 4).



**Figure 3. *C. ternatea* extract (a) indicating the presence of fibers in pigment smear and (b) peeled coating three-quarter grain view (c) TRX-cells<sup>TM</sup> nanolaminae, (d) atop *C. ternatea* fibre (arrows) and, (e) TRX-cell<sup>TM</sup> at the inter-granular spaces (IGS) indicated by arrows on plate (e).**

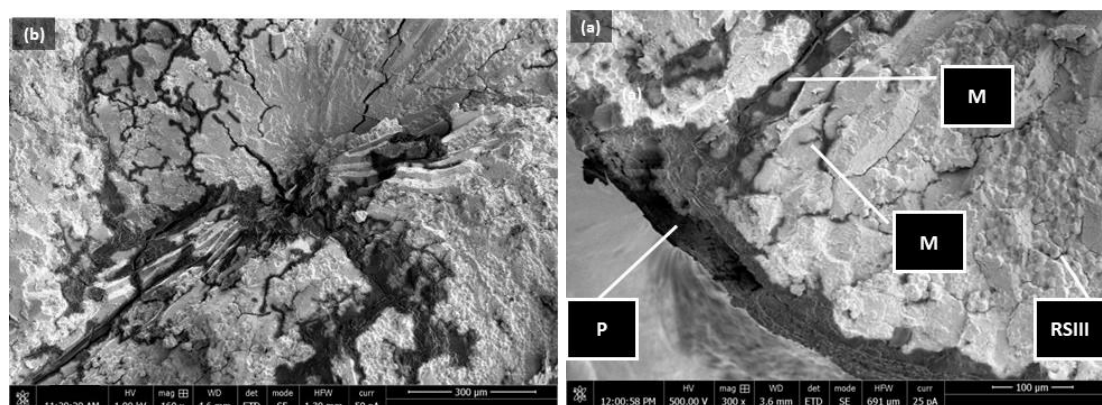
**Delphinidin Affinity for RSIII.** Further analysis of the surface coating required careful dissecting of the grains. While the dried pinacell-coated grains were crispier than the counterpart, the grains coated without pinacell were softer and easier to cut through. The resultant dissections are shown in Figures 4a and 4b. Where nanofibrils could no longer be delineated from the grains, morphological analysis of the pigmented regions of the grains shows evidence of delphinidin penetration beyond 100 µm of the grains' radial depth (Table 2 and Figure 5). As rice surface pores and pinacell compatibility (Figures 4 and 5) revealed a 1:1000 (2-10 µm: 10-90 nm) ratio of rice pore-to-fibril diameter, the penetration profile could be attributable to the nanofibrils, to a certain extent. Considering the limited length of the

fibrils, other mechanisms responsible for pigment penetrations were plausibly capillary action and the possible higher affinity of the delphinidin to the type three resistant starch (RSIII) and the proteinaceous component of parboiled rice grains cf. delphinidin-RAMOL (radical-modified lignin) and delphinidin-cellulose II structure of TRX-cells™. The sophisticated chemistry of anthocyanin [9] was the driving factor for delphinidin's ultimate diffusion to the grain core.



**Figure 4. Pigment-coated parboil grains (a) with and (b) without pinacell and their corresponding pigmented surface cross-section.**

Through the electromagnetic lenses of XHR-FESEM and its Back-scattered Electron (BSE) detector's atomic number sensitivity, the outcome of the more pronounced delphinidin penetration is portrayed by the dark-tone areas in Figure 5. Pigment bleed along the canal was the feature typically encountered in the parboiled grains coated with pinacell. Without added coloring effects, XHR-FESEM assisted in delineating the regions of differing chemical compositions. The convergence of the high and low-energy electron beams promoted spatial resolution while minimizing the charging effect on non-conductive specimens like the pigment-grain barrier.

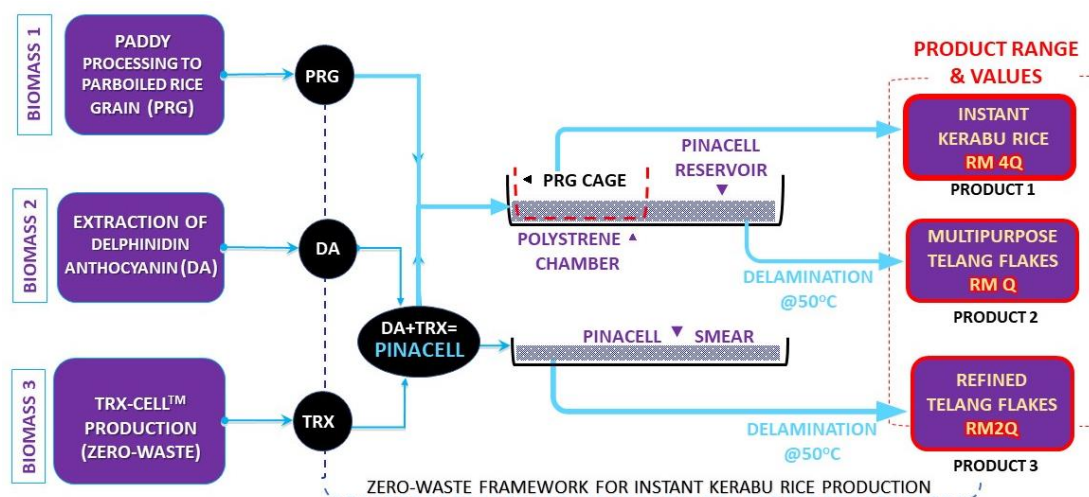


**Figure 5. FESEM of PRG cross-section showing (a) peeled pinacell and pigment diffusion along the micro-cracks and (b) pigment diffusion to the grain core.**

**Longevity by Antisepsis to the Core.** Since the pigments also possessed antibacterial properties, the deep penetration possibly engendered by TRX-cells<sup>TM</sup> or the stronger affinity of the delphinidin anthocyanin to the type three resistant starch (RSIII) also provided an additional antimicrobial effect on the grains, which helped enhance the grain's longevity. Better control of microbial effects also means a successful measure to preserve the nutritional and medicinal values. Sacrificing the therapeutic effects means defeating *C. ternatea* pigment function for zeroing the rice glycemic index, which in turn reduces the potential cause for a spike in blood sugar, the risk for cholesterol, and type 2 diabetes [13] inflammatory bowel disease [23, 24].

Despite low-temperature color stability, the pigment immobilized on TRX-cells<sup>TM</sup> portrays a more striking and uniform color due to the nano-enabling effect of TRX-cells<sup>TM</sup> on pigment spreading. The health benefits and the overall simplicity of food preparation from pinacell flakes and pinacell-coated grains make it a potentially practical *nasi kerabu* and other exotic food preparation despite a hectic schedule.

**Zero-waste Framework.** Analysis of the PRG coating process revealed three potential commercialization candidates; *Instant Kerabu Rice*, *Refined Telang Flakes*, and *General Purpose Telang Flakes* (Figure 6). The side-products were obtained by evaporation at 40°C that initiated dry-flaking or delamination of the pinacell spent liquor spread on a polystyrene surface of the chamber. The phenomenon was due to the higher affinity of delphinidin anthocyanin towards TRX-cells<sup>TM</sup> components like RAMOL, cellulose I and cellulose II compared to the hydrophobic surface of polystyrene. Understanding the underlying science allows zero-waste management of the instant *kerabu* rice production. The findings point to a sustainable process design that takes well-being and responsible resource consumption seriously, in line with the United Nation's Sustainable Development Goals 3 and 12 (SDG3 and SDG12).



**Figure 6. Framework for zero-waste production of Instant Kerabu Rice and delphinidin anthocyanin flakes for commercialization.**

## Conclusion

Pigment immobilization on the nanometer scale cells (pinacell) registered as TRX-cells™ allowed better color uniformity and aesthetic value, crucial in consumers' first impression. The extended shelf life of the pinacell-coated grains is a resounding factor for commercialization, and the pinacell enabled the feature. Longevity, a vital feature to comply with transportation and storage logistics, is critical in product marketability. Efficient pigment penetration offering dual effects (antimicrobial and ultimate quality preservation) is also critical for preventing wastage of the antioxidant pigment during rice cooking, particularly steaming. The ease of delaminating pinacell spent liquor on polystyrene surface signals an excellent promise for zero-waste *Instant Kerabu Rice* production. The maximized pinacell-polystyrene surface tension avails the possibility of commercializing two grades of *C. ternatea* delphinidin flakes as a natural coloring agent. It is noteworthy that the beneficial stated outcome could not be possibly understood without the atomic sensitivity of XHR-FESEM, which allowed us to understand the way pinacell effect precision parboiled rice coating with *C. ternatea* delphinidin anthocyanin.

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## Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

## Disclosure of conflict of interest

The authors have no disclosures to declare.

## Compliance with ethical standards

The work is compliant with ethical standards.

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