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Thermotolerance of Immobilized *Lactobacillus plantarum*-Alginate-Palm Kernel Cake Bead as Potential Ruminant Feed Pellet

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ABSTRACT

The development of a thermal protective technique in the feed manufacturing process is crucial for delivering a sufficient number of probiotics. This study was aimed to determine an optimum ratio between a combination of heterogenous encapsulation of palm kernel cake (PKC) and sodium alginate (Al) for immobilized Lactobacillus plantarum ATCC 8014 survivability and its thermo-tolerance. In this study, bead particles at a ratio of 3:2 (Al: PKC) gave the most efficient encapsulation, which produced the highest cell viability (70.39%). Similarly, this ratio also showed higher cell survivability (97.51%) upon simulated heat exposure. Fourier Transform-Infrared Spectroscopy (FT-IR) confirmed the changes in the functional bonds in the presence of PKC, probiotic, and alginate. The thermogravimetric analysis also showed the 3:2 bead ratio gave the best thermal protection rate at 84.06%. Thus, this newly developed heterogeneous bead could be applied as a value-added additive in the ruminant pellet industry to significantly impact ruminant growth and health.

1. Introduction

The inclusion of probiotic bacteria in animal feed has become an alternative strategy to substitute antibiotic growth promoters (AGP). However, in terms of practicality, the administration of AGP in livestock is costly, laborious, and in certain conditions, may interfere with the activity of digestive enzymes. Moreover, concerns are raised over its impact on the increasing cases of antibiotic resistance, particularly resistance to gram-negative bacteria (Salmonella spp. and Escherichia coli) that can trigger severe problems in treating infectious diseases [1]. In contrast, there are many benefits to pelleting animal feed with the probiotic bacteria; these include improvement of animal

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weight gain, strengthen digestibility, increase in nutrient intake, decrease of energy expenditure for food consumption, and prevention of enteric pathogenic infection [2]. Furthermore, this pelleting process has a wide range of conditioning temperature and retention time, essential for the commercialization of feed mills [3]. Alternatively, in the current industry practices, the most used conditioning temperature in the feed mills may reach up to 90^{o}C, consequently damaging certain heat-sensitive nutrients and killing many probiotics [3, 4]. Therefore, the addition of heat-protective agents is suggested to enhance strain survival during pelleting and drying processes [5].

In the development of probiotic strain for feed additives, there are a few criteria to be considered such as unharmful to host, stable in gastric acid or bile, able to survive in the gastrointestinal transit, able to synthesize the antimicrobial substances, adhesion to epithelial cells, able to inhibit pathogenic bacteria, resistance to antibiotics, tolerance to food additives and stability in the food matrix [6]. The mode of action for probiotics is highly dependent on the type of enteric bacteria and the severity of diseases to be treated [7]. In some mechanisms of probiotics, they adhere to the intestinal mucosal surface, which prevent the colonization of pathogenic bacteria [7]. Three strains of probiotic bacteria isolated from dairy cows' feces, which are Lactobacillus gasseri, Lactobacillus reuteri, and Lactobacillus salivarius, were found to be able to inhibit pathogenic bacteria such as *Escherichia coli* O157:H7, *Mycobacterium avium* subspecies *paratuberculosis*, and Salmonella species (*Salmonella enteritidis*, *Salmonella typhimurium*, and *Salmonella Dublin*) [8]. These specific probiotic strains could favor treatment of five infectious disorders, namely necrotizing enterocolitis, acute infectious diarrhea, acute respiratory tract infections, antibiotic-associated diarrhea, and infant colic infectious [9]. Hence, the application of probiotics in feed pellets to treat infectious diseases in ruminants has been regarded as a suitable alternative for replacing traditional antibiotic therapy.

Thermostability of probiotics is one of the critical technological challenges in the feed manufacturing process due to the exposure of feed pellet to ultra-high temperature processing such as pelleting, extrusion, cooking, steam flaking, conditioning, expanding, roasting, popping, toasting, and micronization that may degrade the feed pellet's heat-sensitive nutrients and kill its probiotics. Encapsulation technique is a feasible technological approach to retain nutrients and preserve viable probiotics in the food industry. An entrapment of nutrients can be developed via surface coating technique using biopolymer materials such as alginate, chitosan, gelatin, whey protein, cellulose derivatives, and many others [10]. Alginate is widely used to encapsulate living cells, including probiotics, because of its unique properties such as water retention, gelation capacity, and stabilizing properties [11]. However, most probiotics may lose their activity and exhibit a high death rate due to thermal inactivation at a temperature of more than 60^{o}C. Various dehydration methods are used to overcome the poor thermostability in probiotics including freeze-drying, sublimation drying, fluidization drying using inert materials (carriers), and spray drying [12]. Freeze-drying is a conventional drying technique commonly used to maintain the viability of probiotic culture for long-term storage. Although it is more convenient and accessible as it does not require freezing conditions during distribution, it is lengthy and more expensive than any other drying processes [13, 14].

Since several decades ago, the palm oil industry is considered a primary biodiesel contributor in Malaysia. The expansion of this industry has led to excessive production of palm oil processing mills and empty fruit bunch. Excessive production of palm oil causes the leaching out of vast amounts of contaminated and toxic materials to the surface runoff, followed by their flow to the oceans, and the later toxification of marine life [15]. The considerable accumulation of PKC poses a significant threat to climate change due to the emission of greenhouse gases such as carbon dioxide (CO_2), methane (CH_4), water vapor (H_2O), nitrous oxide (N_2O), and ozone (O_3) [15]. One mitigation strategy to reduce the abundance of solid waste disposal from the palm-oil cake is the nutrient recycling of PKC for the broiler and animal feed industry. Very similar to the corn gluten or rice bran, the high crude fiber (13-

20%) and protein content (16-18%) in the PKC is the preferable choice for energy to meet the requirements of ruminants. Nevertheless, it is considered as a higher value for non-ruminant [16].

To the best of our knowledge, there is a lack of information on the heterogeneous encapsulant thermal characteristics that could enhance probiotic survivability during the pelleting process. This study was conducted to address the stability and efficacy of the freeze-drying of microencapsulated palm kernel cake with alginate upon exposure to different thermal heat. The objectives was to determine relationship between heterogeneous encapsulant characteristics of palm kernel cake (PKC) and sodium alginate (Al) with immobilized Lactobacillus plantarum ATCC 8014 survivability and its thermo-tolerance.

2. Materials and Methods

2.1 Bacteria Culture

1 % of Lactobacillus plantarum ATCC 8014 inoculum was cultured in sterile de Man, Rogosa, and Sharpe (MRS) broth and incubated overnight at 37^{o}C. After that, the cells were centrifuged and washed twice with sterile peptone water.

2.2 Preparation of Fiber

Palm Kernel Cake (PKC) was cut into small pieces. After that, the dirt at the surface of PKC was cleaned under running tap water. After that, the PKC was dried at 60^{o}C for 24 h. Then, the dried PKC was ground and sieved using a micro-sieve shaker (Endecotts, UK). The fine powder (150-250 µm) was kept in an air-tight bottle before microencapsulation.

2.3 Preparation of Microencapsulation

Sodium alginate solutions at 1-3% (w/v) were prepared, sterilized at 120^{o}C for 15 min, and cooled at 38-40^{o}C. Next, the sterile sodium alginate solution was dissolved in two different weights of palm kernel cake (PKC) (2 g and 4 g, respectively) to varying ratios of Al: PKC (1:2, 2:2, 3:2, 1:4, 2:4, and 3:4, respectively). Then, probiotic-loaded capsules were prepared by mixing 8 mL of cell suspensions into the PKC as a source of fiber. After that, 100 mL soybean oil with 1% tween-80 was added to the beaker. Next, the probiotic-loaded capsules were added dropwise while stirring magnetically in a beaker. After 5 min, a uniform turbid emulsion was obtained. Then, the sample was pipetted using a 10 mL syringe into 100 mL of 0.1 M calcium chloride to harden the encapsulated beads. Finally, beads were separated by filtration using Whatman filter paper (No. 1) (GE-healthcare, UK), and transferred to a sterile petri dish. The samples were stored in a refrigerator at 7±1^{o}C until further analysis.

2.4 Freeze Drying Method

The microencapsulated beads were freeze-dried at 40^{o}C for 24 h to produce dried samples. Then, the freeze-dried samples were packed in polyethylene bags, sealed in aluminum foil, and stored at 4^{o}C until further analysis.

2.5 Microencapsulation Efficiency

The probiotic powder (1 g) was dissolved into 10 mL of 1% peptone solution. Serial dilution of the probiotic mixture was prepared, and plating was performed on MRS agar. The plates were incubated

at 37^{o}C for 48 h. Then, colony-forming units of Lactobacilli bacteria were counted to determine the survival rate. The best three formulations from the microencapsulation efficiency result were selected to analyze simulated heat exposure further and simulated in vitro release.

2.6 Simulated Heat Exposure

Approximately 1 g of encapsulated probiotic bead particles were transferred into test tubes containing 10 mL of peptone. Then, the solution was pre-heated at 90^{o}C for 30 s. After that, the solution was cooled immediately in cool water at room temperature (25^{o}C). Next, the viable counts of the microencapsulated probiotics powder were performed.

2.7 Analysis of Fourier Transform-Infrared Spectroscopy (FT-IR)

The direct scanner method was applied in the analysis, where the probiotic sample was placed on the top of the crystal Nicolet iS10 FT-IR Spectrometer (Thermo-Scientific, USA). Then, the clamp was tightened down to hold the sample. Next, infrared light was bounced onto the crystal plate, and FT-IR spectra were recorded by averaging 16 scans per sample from 4000 to 600 cm^{-1} at a resolution of 4 cm^{-1}.

2.8 Microscopy Examination

A small quantity of the powder was spread on a slide. Then, a few drops of glycerol solution were added. After that, the slides were examined under 400X magnification using a compound microscope (Model: OM159 40X-1000X; Omano, USA).

2.9 Thermogravimetric Analysis (TGA)

The probiotic powder was analyzed by using a thermogravimetric analyzer (Mettler-Toledo, Ohio, USA). First, approximately 1-5 mg of bead particles were placed on the ceramic sample holder. Then, it was heated up to 700^{o}C under nitrogen gas at a flow rate of 20^{o}C/min. Finally, the sample was cooled down at room temperature to ensure the completion of the heating process.

2.10 Statistical Analysis

Data obtained were analyzed to compare statistical significance at 0.05 using the One-way Analysis of Variance and Duncan Multiple Range Test. The analysis was done using software IBM SPSS Statistics (64-bit edition).

3. Results and Discussion

3.1 Viability of Coated Heterogeneous Beads After Encapsulation, Thermal Exposure, and In-vitro Delivery

3.1 Encapsulation Efficiency

Higher encapsulation efficiency was obtained using 2% fiber-derived PKC, as compared to 4%. Furthermore, when the proportion of alginate was increased (1-3%), the efficiency also increased

(65.41%, 66.89%, and 70.39%, respectively) (Table 1). In contrast, lower efficiency was recorded when bead particle ratios at 1:4, 2:4, and 3:4 (58.82%, 63.26%, and 65.95%) were used.

Table 1
Encapsulation efficiency at different bead particle ratios between Alginate (Al) and fiber-derived PKC (F)

Bead particle ratio (Al: F)	Encapsulation efficiency (%)
1:2	65.95[c]
2:2	66.89[d]
3:2	70.39[e]
1:4	58.82[a]
2:4	63.26[b]
3:4	65.41[cd]

[abcde]Different letters within a column indicate a significant difference *(P≤ 0.05).*

At a ratio of 3:2 (Alginate to fiber-derived PKC), the bead particle was suggested to have obtained the highest encapsulation efficiency. The result indicates that the higher percentage of fiber-derived PKC has reduced the encapsulation efficiency due to the inefficient encapsulation process of PKC into alginate matrices. Thus, it affected the amount of cell survivability. As a result, it shows zero encapsulation efficiency for the bead particles without fiber. Based on previous findings, it was reported that the incorporation of sugarcane bagasse enhanced the ability of probiotics after the encapsulation process compared to samples without fiber [17]. It was proven that the immobilization of L. plantarum on PKC before the encapsulation could support a retention period of cell viability and increase efficiency [17]. In addition, the incorporation of fiber provided a larger surface area for the attachment of probiotics before the encapsulation process occurred. Furthermore, the use of alginate was able to trap the PKC matrices and immobilized probiotics. The PKC entrapment enhances the probiotic's survivability inside the bead particles [18]. The higher efficiency of encapsulation of probiotics was also due to the non-toxic alginate. However, the loss of cell viability may slightly affect the extrusion during the encapsulation process.

3.2 Survivability of probiotic cell of L. plantarum after simulated heat treatment

The best temperature and duration for the pelleting process were obtained at 90^{o}C for 30 s (Table 2). The bead particles incorporated with 2% PKC were selected for simulated heat treatment due to their high encapsulation efficiency. Cell survivability after simulated heat exposure showed that the bead particle ratio of 3:2 has a higher number of cell viability (97.51%) compared to the encapsulant bead particles ratios of 2:2 (96.58%) and 1:2 (92.80%).

Table 2
Survivability of probiotic cell of L. plantarum after simulated heat treatment process on bead particles at different ratios of Alginate (Al) and 2% fiber-derived PKC (F)

Sample	Cell survivability (%)
Free probiotic without immobilization (control)	35.02[a]
Immobilized probiotic cell at 1Al:2F	92.80[b]
Immobilized probiotic cell at 2Al:2F	96.58[c]
Immobilized probiotic cell at 3Al:2F	97.51[d]

[abcd]Different letters within a column indicate a significant difference *(P≤0.05)*.

Heat exposure reduced the cell viability of immobilized L. plantarum with PKC, which is highly dependent on alginate concentration. The higher the concentration of alginate, the lesser the free volume in the encapsulated bead. It reduces the permeability of heat due to the formation of complex encapsulant matrices. According to [19], slow diffusion of hot water leads to higher survival of probiotics after heat exposure. Therefore, the reduction occurs during heat transfer via a complex layer of alginate from layer to layer. When the alginate concentration increases, it can help to increase the cell survivability during heat exposure. The different concentrations of alginate may influence cell survivability during simulated heat exposure. However, for the free cells of L. plantarum, it was found to be sensitive to heat as its viability was reduced up to 65%. Furthermore, all the samples could protect the cell survivability even after higher heat exposure as the heat intensity was scattered to all areas of solid particles of the fiber (PKC). Consequently, it reduces the heat exposure to the immobilized probiotic. According to [17], immobilized probiotic incorporated with sugarcane bagasse particles under a scanning electron microscope shows that the fiber helps reduce the heat intensity when its directly exposed to the probiotics. These findings were supported by [20]. They suggested two alternative ways to enhance thermo-tolerance of the cell microencapsulation, which are through the application of an increased concentration of encapsulant agent, or secondly, the incorporation of biomaterials with the encapsulant agent. The result obtained shows that alginate is one of the best encapsulation agents compared to other types of biomaterials.

3.3 Surface Morphology of Alginate to Fiber-derived PKC

The effects of different ratios of alginate to PKC on surface morphology are illustrated in Figure 1. It was observed that an increase in percentage of alginate (1-3%) without the presence of PKC affected the surface pore size, which became tiny, microscopic. However, the structure inside the solid particle was recorded to be loosened (Figure 1(a)). In contrast, with the presence of PKC fixed at 2%, a solid particle formed a dense vesicle and granular due to the intact structure of the fiber inside the immobilized probiotic ((Figure 1(b)). Thus, the morphology of the bead particles at ratio 3:2 exhibited a potential of a denser surface compared to ratios 1:2 and 2:2.

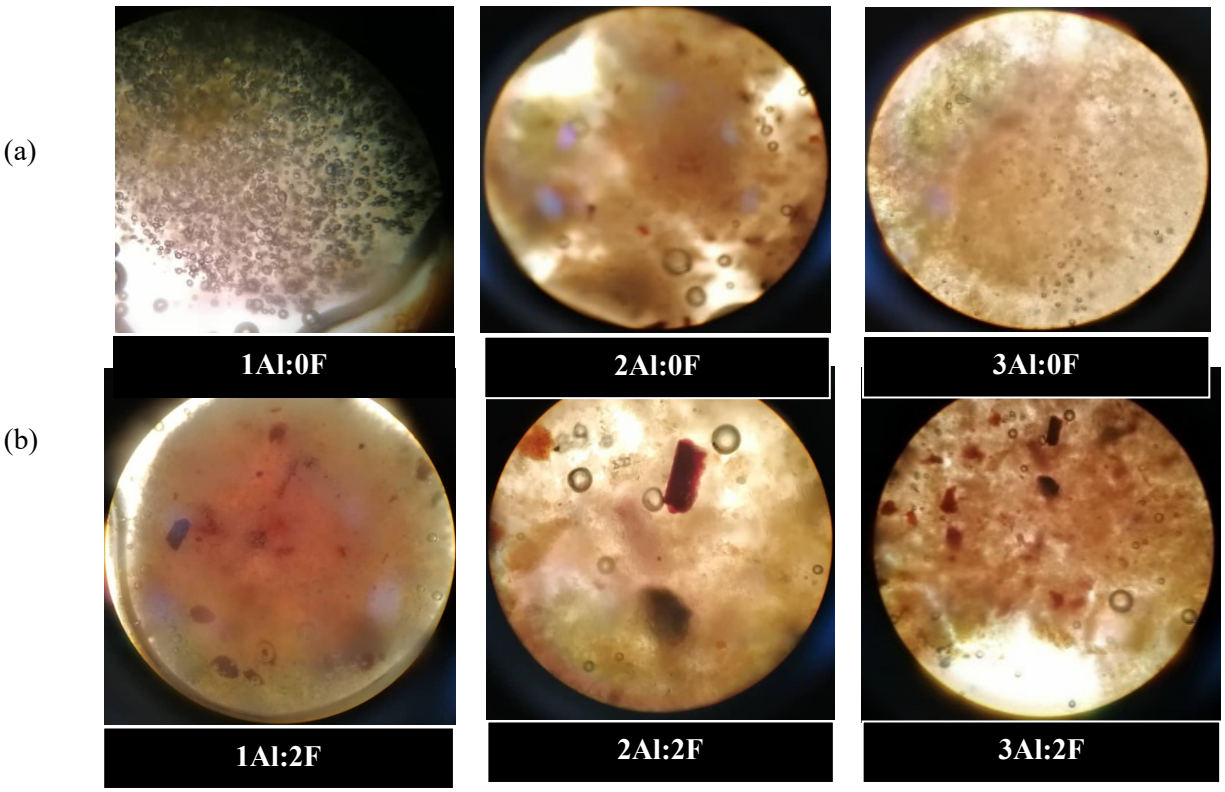

Fig. 1. Surface morphology image of bead particles showing (a) immobilized L. plantarum without PKC (control sample) at 1-3% of alginate, and (b) immobilized L. plantarum with 2% fiber-derived PKC (F) at 1-3% of alginate by using microscope compound at 400X magnification

3.4 Biochemical Characterization of PKC using FT-IR

As shown in Figure 2, the FT-IR spectra revealed several spectra that indicated components on the bead particles. Biochemically, PKC is a lignocellulosic fiber consisting of cellulose, hemicellulose, and lignin. PKC spectrum shows a broad wavelength band at 1008.67 cm−1, which is attributed to CO-O-CO stretching vibrations. In theory, when a FT-IR peak appears at 1743 cm−1, it represents the hemicellulose carbonyl bond (C=O).

According to [17], if a peak appears at 1236 cm−1, it is attributed to the C-O-C stretching vibration in the cellulose chain. Alternatively, the FT-IR spectra of L. plantarum showed a spectrum at peak 2940.12 cm^{-1}, indicating C-H stretching. Interaction between L. plantarum and PKC shows that the spectrum peak shifted to a different peak range for all the samples of bead particles ranging from 3004.63 to 3004.68 cm^{-1}. This phenomenon may have occurred due to the probiotics that covered the entire regions of the PKC's surface. This causes the peak value to slightly shift, reflecting the combination of C-H strength with anhydride group [17]. In contrast, the interaction between alginate and PKC at the four respective ratios (1:0, 3:0, 1:2, and 3:2) shows that the absorption value was recorded at a range of 1376.67 to 1376.85 cm-1, which represented the C-H bending. The characteristic peak of alginate was seen in the calcium alginate fiber spectra at 1644 cm^{-1}, corresponding to carbonyl (C═O) bond or carbonyl stretching in amide and amino group at the wavelength band of 1173 cm−1 [21].

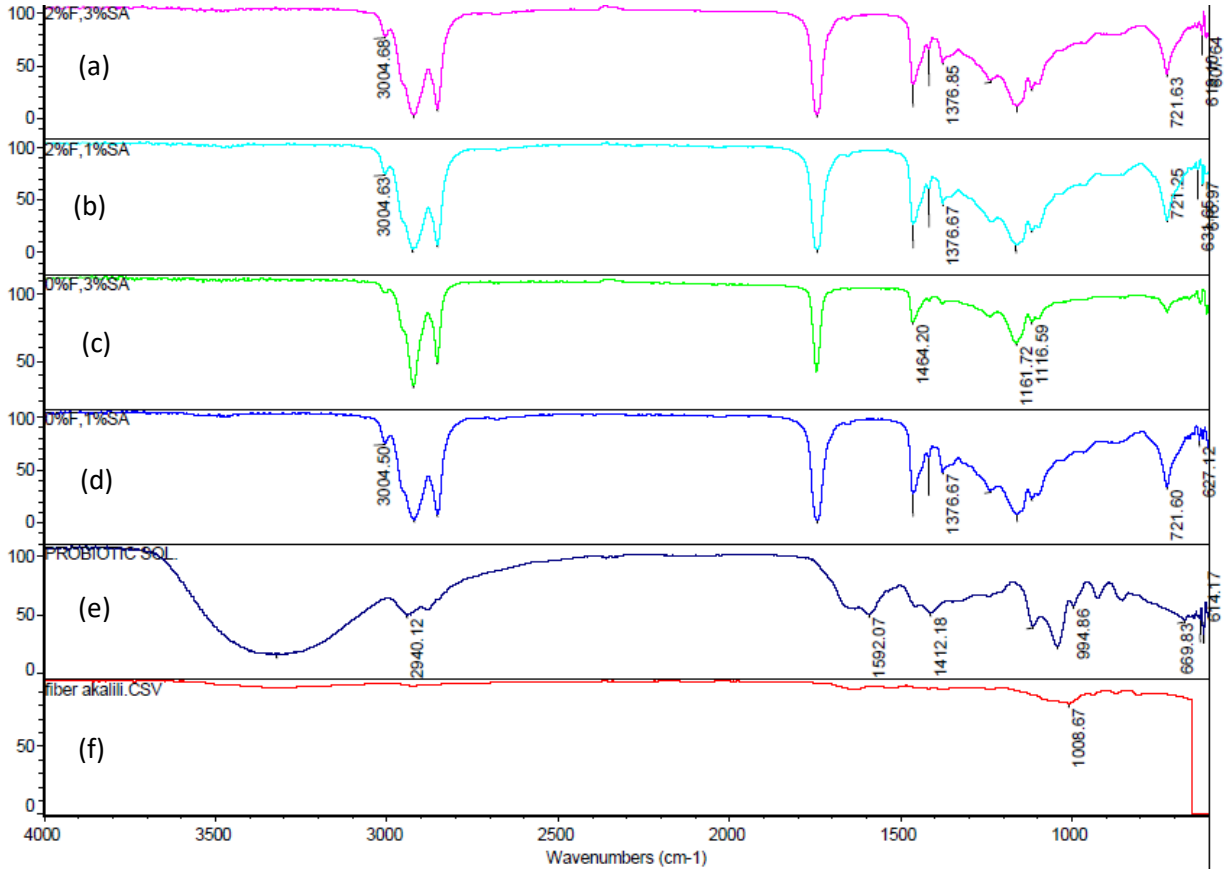

Fig. 2. FT-IR spectra of bead particle showing (a) encapsulated with 3:2, (b) encapsulated with 1:2, (c) encapsulated with 3:0, (d) encapsulated with 1:0, (e) free probiotic cell, and (f) fiber-derived PKC only. Abbreviations Al denotes alginate, and F represents fiber-derived PKC

3.5 Thermogravimetric Analysis (TGA) of Bead Particles

In this study, nine samples were heated from room temperature (25^{o}C) to 700^{o}C at a rate of 20^{o}C/min. TGA curves are illustrated in Figure 4. Based on Figure 3, all the bead particles incorporated with different percentages of fiber and alginate decomposed in a similar four-step degradation process, corresponding to the four-temperatures range (30-130^{o}C, 130-360^{o}C, 360-440^{o}C, and 440-700^{o}C, respectively). In the first stage of degradation (30-130^{o}C), about 3% of the weight was lost from the sample due to the loss of microbial particles. However, based on the final percentage of residues that were obtained from all the samples, it was observed that the bead particles with the ratio of 2:2 and 3:2 have a higher percentage value of residues (56.31% and 84.06%, respectively), in comparison to the bead particles with the ratio of 1:0, 2:0, 3:0, 1:2, 1:4, 2:4 and 3:4 that ranged from 1.86% to 9.39%.

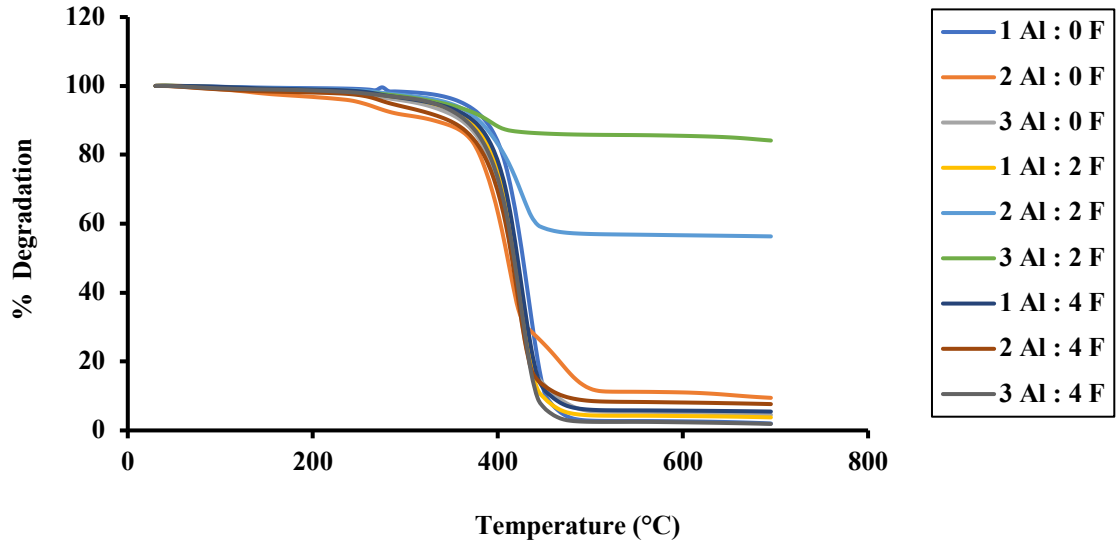

Fig. 3. TGA curves showing levels of degradation upon heat exposure at four-temperature range (30-130^{o}C, 130-360^{o}C, 360-440^{o}C, and 440-700^{o}C, respectively) for nine combinations of bead particle ratio between alginate (Al) and fiber-derived from PKC (F)

In this case study, the microbial sample lost from the bead particle was the L. plantarum, which has been hypothetically stated to be destructed at 70^{o}C [22]. The loss was seen at the initial stage of degradation, indicating the degradation of bacteria. In the second stage of degradation (130-360^{o}C), about 9% of the weight was lost from the sample due to water loss in the bead particles. Theoretically, the drying period usually occurs when the temperature is above 100^{o}C because light volatiles such as water particles will be lost during this phase [23]. At the third stage of degradation (360-440^{o}C), about 10-90% of the weight was lost due to the thermo-chemical conversion process involving biomass. Consequently, the final for the degradation of fiber (PKC) occurred because the main chain of carbon has been decomposed. This stage of decomposition is usually known as the significant slope of the TGA curve. It corresponds to a substantial drop in sample weight due to the liberation of volatile hydrocarbon of hemicellulose, cellulose, and some parts of lignin [22]. The last stage of degradation was at 440 to 700^{o}C, about 0.5-2.5% of weight was lost from the sample. This weight loss was not as influential as in the initial stage due to the decomposition of the remaining component, which was alginate. In addition, the results show the varied range of weight loss in each stage due to the different concentrations of alginate for each sample. The encapsulation of probiotics with PKC coated with alginate has improved the thermal degradation of the beads. Simulated heat exposure analysis shows bead particles with ratios of 2:2 and 2:3 have higher cell survivability. Thus, it was proven that bead particles with ratios of 2:2 and 2:3 were the best ratios to be used in the thermal protective technique of the bead particles.

4. Conclusions

The best ratio for the encapsulation of feed pellet production in thermal protective was at 3:2 (Al:PKC), with a probiotic survivability of 97.51%. In addition, this ratio can promote the highest probiotic in vitro simulated release at 7.44 $\log_{10}$CFU/mL. Therefore, this study is beneficial for producing probiotic-ruminant feed pellets to prevent probiotic degradation due to high heat treatment and higher targeted delivery of probiotics to the host.

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