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The Effect of Inlet Temperature on Starter Culture Properties of Spray-Dried *Pediococcus acidilactici*

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Laurentius Hardy Kurniawan
19010089

Putu Virgina Partha Devanthi, S.Si., M.Si., Ph.D. (i3L/Field Supervisor)

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RESEARCH REPORT

THE EFFECT OF INLET TEMPERATURE ON STARTER CULTURE PROPERTIES OF SPRAY-DRIED *Pediococcus acidilactici*

By
Laurentius Hardy Kurniawan
19010089

Submitted to

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Research Project/Field Supervisor: Putu Virgina Partha Devanthi, S.Si., M.Si., Ph.D.

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CERTIFICATE OF APPROVAL

INSTITUT BIO SCIENTIA INTERNASIONAL INDONESIA

Jl. Pulomas Barat Kav. 88 Jakarta Timur 13210 Indonesia
+6221 295 67888, +6221 295 67899, +6221 296 17296
www.i3l.ac.id

Certificate of Approval

Student	: Laurentius Hardy Kurniawan
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1	Thesis Supervisor	: Putu V.P.D. S.Si., M.Si., Ph.D.	Approved
2	Lead Assessor	: Adinda D.K. , S.Si., M.Si., M.Eng., Ph.D.	Approved
3	Assessor 2	: Dr. Riahna B.K. , S.Si., M.Sc.	Approved

Acknowledged by,

Head of Study Program,

Ihsan Tria Pramanda, S.Si., M.Sc.

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ABSTRACT

Functional foods, including fermented foods, have gained an increasing demand recently due to the consumers' awareness of the health benefits offered. To produce fermented food, a starter culture is required. *Pediococcus acidilactici* has recently gained research attention due to its potential starter culture and probiotic properties. Commercial starter cultures are commonly present in a dry form, and spray drying is a common method used for producing stable and long shelf-life dry cultures. However, spray drying involves a very high inlet temperature which leads to heat and osmotic stress that may damage the cells' cell wall, DNA, and metabolic activities. However, the spray drying effect on *P. acidilactici*'s starter culture properties has not been studied. Therefore, spray drying with different inlet temperatures (120^{o}C, 150^{o}C, and 170^{o}C) were performed in this study to investigate its effect towards the *P. acidilactici*'s starter culture properties, including milk coagulation, acidification, and proteolytic properties. The results showed that all samples still retained the starter culture properties. Samples had 42.16 to 42.28% curd yield and 3.90 to 4.49 mg/mL soluble protein left from milk coagulation, pH of 5.38 to 5.49, and proteolytic index ratio of 0.57 to 0.67. However, different inlet temperatures did not have a significant effect ($P>0.05$) towards the properties. This finding suggests that spray drying of *P. acidilactici* is considerable as a method to produce starter culture powder in the future.

Keywords: *Pediococcus acidilactici*, starter culture, spray drying, encapsulation.

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To whoever is reading this and working on your research, do not give up and stay strong as there will always be answers to everything.

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LIST OF ABBREVIATIONS

CFU	Colony forming units
FAO	Food and Agriculture Organization of the United Nations
LAB	Lactic acid bacteria
MRS	de Man Rogosa Sharpe
NS	Not significant
OD	Optical density
pH	Power of hydrogen
PIR	Proteolytic Index Ratio
RPM	Rotations per minute
WHO	World Health Organization

I. INTRODUCTION

1.1. Background

According to FAO/WHO (2002), probiotics are living microorganisms that provide health benefits towards the host when administered in adequate amounts. They are known for their abilities to help the digestion process, boost the immune system, maintain the gut flora, improve atopic eczema, provide hypocholesterolemic effect, and reduce the risk of colon cancer (Shi et al., 2016; Williams, 2010). Probiotics are commonly found in the form of supplements. However, they are also commonly found in fermented foods as they are utilized as starter cultures (Dinkçi, Akdeniz, & Akalin, 2019).

Starter cultures are living microorganisms added to food to initiate food fermentation (Laranjo, Potes, & Elias, 2019). The common properties of starter culture are acidification, coagulation, proteolytic activities, and carbohydrate metabolism (Abbasiliasi et al., 2017). In addition, a starter culture with probiotic properties greatly benefits the host's health (Mafra et al., 2020). The lactic acid bacteria (LAB) group is a common starter culture source with probiotic properties, such as from *Lactobacillus and Bifidobacterium* species. Recently, *Pediococcus acidilactici* has gained popularity in research due its potential use as starter culture and probiotic (Barigela & Bhukya, 2021; Holland, Crow, & Curry, 2022; Olajugbagbe, Elugbadebo, & Omafuvbe, 2020; Song et al., 2021). As a starter culture, *P. acidilactici* has the ability to ferment carbohydrates and proteins, produce lactic acid, and coagulate milk (Abbasiliasi et al., 2017; Wang et al., 2019). *P. acidilactici* has been isolated from fermented foods such as comté, Wara, *terasi*, *peda*, *bekasam*, *petis*, and *tempoyak* (Olajugbagbe, Elugbadebo, & Omafuvbe, 2020; Purwati, Kurnia, & Pratama, 2019; Pramono, Murwantoko, & Triyanto, 2013; Yuliana & Garcia, 2009).

The combination of probiotic and starter culture properties of *P. acidilactici* has a high potential for producing functional foods which provide additional health benefits (Damián et al., 2022). The increasing health cost, including medicines and treatments, has made health a treasure. Due to that, consumers are more conscious of maintaining their health as one of the methods to prevent diseases. This phenomenon leads to the increasing demand for functional food, thus growing the market size (Ali & Rahut, 2019; Topolska, Florkiewicz, & Filipiak-florkiewicz, 2021).

Both starter cultures and probiotics are commonly dried to produce an easy-to-use product/ingredient, improving its stability and shelf-life and reducing transportation costs. Spray drying is a common technique used to dry cultures due to its high productivity and cost-effectiveness. Spray drying works by spraying the targeted solution into hot and dry air, with temperatures ranging from 150°C to 250°C. The moisture is instantly removed, leaving fine solid

particles between 10 to 150 µm. However, spray drying may influence the cell's viability and metabolic activity due to heat stress, osmotic stress, and desiccation (Huang et al., 2017).

Previous studies have reported that thermal stress during spray drying might also affect the probiotics' functionality, and the effect is not directly linked to cell survival. Furthermore, the heat may cause damage to the cell wall, RNA, and DNA of the cells, disrupting their metabolic activities (Ortega, 2017; Perdana et al., 2013; Tripathi & Giri, 2014). Hence, affects their abilities as a starter culture. Therefore, this study aims to investigate the starter culture properties, including the coagulation, acidification, and proteolytic properties of *P. acidilactici* after spray drying using different inlet temperatures. The medium used in this study was milk and skim milk, which are the common model used in similar studies related to starter culture properties (Akabanda et al., 2014; Cellesi et al., 2019; Li et al., 2022; Popović et al., 2020; Wardani et al., 2017).

1.2. Research Questions

Based on the background, the research questions are formulated:

1. Do different inlet air temperatures in spray drying affect the milk coagulation property of *P. acidilactici*?
2. Do different inlet air temperatures in spray drying affect the milk acidification property of *P. acidilactici*?
3. Do different inlet air temperatures in spray drying affect the proteolytic property of *P. acidilactici*?
4. Which inlet air temperature results in the best starter culture properties?

1.3. Hypothesis

These are the hypotheses related to the research questions:

1. H_0: different inlet temperatures applied for spray drying will not affect the coagulation properties of *P. acidilactici* significantly.

   H_1: significant differences in coagulation properties of spray-dried *P. acidilactici* will be observed between different spray drying inlet temperature samples.
2. H_0: different inlet temperatures applied for spray drying will not significantly affect the acidification properties of *P. acidilactici*.

   H_1: significant differences in acidification properties of spray-dried *P. acidilactici* will be observed between different spray drying inlet temperatures samples.
3. H_0: different inlet temperatures applied for spray drying will not significantly affect the proteolytic activity of *P. acidilactici*.

H_1: significant differences in the proteolytic activity of spray-dried *P. acidilactici* will be observed between different spray drying inlet temperatures samples.

1.4. Research Scope

Research scopes of this research include:

1. Culture preparation of *P. acidilactici*.
2. The spray drying process in different inlet temperatures (120^{o}, 150^{o}, and 170^{o}C) to produce spray-dried *P. acidilactici* powder.
3. Characterization of starter culture properties of spray-dried *P. acidilactici* produced from different inlet temperatures using milk as the medium for coagulation and acidification tests, and skim milk agar for the proteolytic test.

II. LITERATURE REVIEW

2.1. Starter Culture

Starter cultures are defined as a single or combination of two or more microbes that perform fermentation processes when added to food, resulting in beneficial compounds or products generated from their enzymatic activities or metabolism. Microorganisms that have huge potential to be starter cultures commonly derived from those that can be isolated from traditional products, in which they show the capability to survive and adapt to the environmental conditions and prevent food spoilage (García-Díez & Saraiva, 2021; Laranjo, Potes, & Elias, 2019). Common properties possessed by starter cultures are acidification, coagulation properties, and proteolytic activity (Abbasiliasi et al., 2017; García-Díez & Saraiva, 2021). Acidification property refers to the ability of the starter culture to produce acids from its metabolic activity, resulting in a drop of pH that can control food pathogens (Ameen & Caruso, 2017). Coagulation refers to the alteration of protein structure from fluidy to a more solid state due to high temperature or acidic conditions, allowing the aggregation of protein molecules (Wu et al., 2021). Both acidification and coagulation properties are commonly related and utilized in the fermentation of dairy products (García-Díez & Saraiva, 2021). Meanwhile, proteolytic activity refers to the hydrolysis of peptide bonds in proteins and has been associated with the organoleptic properties of fermented dairy products (Kieliszek et al., 2021; Mótyán, Tóth, & Tőzsér, 2013).

2.2. Lactic Acid Bacteria (LAB) & *Pediococcus acidilactici*

LAB is a group of bacteria that has been known for their applications in food fermentation (Mathur, Beresford, & Cotter, 2020). They are gram-positive bacteria, rod or cocci in shape, acid-tolerant, and non-spore-forming (Bintsis, 2018). *Pediococcus*, *Lactobacillus*, and *Streptococcus* are LAB commonly used in dairy product fermentation. However, their applications are not limited to dairy products but also include meat, cereal, and vegetable fermentation (Mathur, Beresford, & Cotter, 2020). The broad range of LAB applications is the result of the properties they possessed, including environmental, nutritional, and adhesional adaptations. Organic acids, antimicrobial compounds, polyols, vitamins, exopolysaccharides, and degradation of proteins are some beneficial compounds produced by LAB (Bintsis, 2018). In addition, most of the byproducts from LAB fermentation often provide health-promoting effects, which include anti-oxidant, immunomodulatory, and anti-allergenic effects, among others (Hill et al., 2017; Linares et al., 2017; Martinez, Bedani, & Saad, 2015).

P. acidilactici is one of the LABs which gained research interest due to its huge potential as both starter culture and probiotic. It has a broad range of growth conditions with a pH range of 3 - 8.0, temperature of up to 50^{o}C, and is categorized as facultative anaerobic, but can grow rapidly aerobically (Olajugbagbe, Elugbadebo, & Omafuvbe, 2020; Papagianni & Anastasiadou, 2009). *P. acidilactici* has been isolated from Tarhana (traditional fermented cereal product), traditional Korean Nuruk, Nhang (traditional Thailand fermented beef), Bekasam (traditional Indonesian fermented fish), Wara (Nigerian unripened soft cheese), Comté (French cheese), Appenzeller (Switzerland cheese), and fermented milk, among others (Irmler et al., 2013; Kaya, & Şimşek, 2020; Olajugbagbe, Elugbadebo, & Omafuvbe, 2020; Purwati, Kurnia, & Pratama, 2019; Song et al., 2018; Surachat et al., 2020). The native sources of *P. acidilactici* and varying fermented products show its potential as a starter culture. In addition, *P. acidilactici* also produces pediocin AcH, a class IIa bacteriocin that has the potential to be used as a food biopreservative with high stability and a wide range of antibacterial activity. In addition, *P. acidilactici* has been proven effective against *Listeria monocytogenes*, a food pathogen (Olaoye & Dodd, 2010; Song et al., 2018). Furthermore, the probiotic properties of *P. acidilactici* confer health benefits, especially towards gut health and microbiota (Song et al., 2018).

2.3. Milk Coagulation and Acidification

Milk is known for its role as a source of nutrition that has also been utilized in numerous industries, especially in dairy-based products. Furthermore, fermented milk has been considered a functional food due to the beneficial nutritional and health properties it provides (Hadjimbei, Botsaris, & Chrysostomou, 2022; Shiny & Mishra, 2013). Protein in milk is mainly composed of 20% whey proteins and 80% caseins (Chen et al., 2021). Casein is a family comprising several caseins that are highly hydrated particles, including α_s-CN, β-CN, and κ-CN (Huppertz et al., 2018). Milk coagulation is involved in numerous dairy products production, such as yogurt and cheese. Casein micelle is partially linked with each other by colloidal calcium phosphate nanoclusters (CCP) (Boubellouta, Galtier, & Dufour, 2011). In a solution, milk casein micelles are spread, forming a polyelectrolyte layer that leads to stability due to steric and charge repulsion between protein particles. Furthermore, micellar calcium phosphate plays an important role in maintaining the integrity of the micelle (Dalgeish & Law, 1988). When casein micelles are hydrolyzed, they become unstable and precipitate out of the solution (Chen et al., 2021).

Casein micelles are affected by pH and temperature in numerous ways (Dalgeish & Law, 1988). The primary phase of milk coagulation is micelle destabilization due to proteolytic activity, which breaks down the biochemical bonds of κ-casein. The secondary phase refers to the

aggregation of the destabilized micelles (Pazzola, 2019). The addition of rennet and milk acidification are the most common ways to coagulate milk. Rennet contains a protease called chymosin, which may enzymatically hydrolyze κ-CN, leading to changes in the surface charge of the micelle, resulting in increased hydrophobicity, destabilization, and aggregation of casein micelles (Chen et al., 2021). Milk acidification has been shown to result in complex changes. In principle, CCP linkage is dissolved as the pH drops, which leads to changes in the physicochemical properties of the micelles especially in the pH range of 5.0 to 5.5 (Boubellouta, Galtier, & Dufour, 2011).

2.4. Proteolytic

Protease/proteolytic enzymes are enzymes that are able to hydrolyze peptide bonds (Mótyán, Tóth, & Tőzsér, 2013). Protease itself has been utilized in various fields, especially in food-related products. In brewing and cereal processing, proteases play a role in the mashing stage. In cheese making, proteases are required to coagulate milk, forming curds and separating it from the whey. Proteases are also used in bakeries to modify gluten (Ward, 2011). Proteolytic activity has been shown to be one of the main biochemical pathways involved in surface and flavor development (Chen et al., 2021). According to Attri et al. (2018), there are 60 proteases possessed by *Pediococcus*. *P. acidilactici* has been shown to possess proteolytic activity (Abbasiliasi et al., 2017; Biswas et al., 1991; Tulini et al., 2016).

2.5. Spray Drying & Inlet Temperature

Spray drying is one of the available methods used to preserve cultures that has gained interest recently (Peralta et al., 2017). Spray drying is a common thermal method used to produce powder from liquid, in which encapsulation materials are also utilized to prevent a disruption in the functionality of the targeted product due to the spray drying environment (Chen et al., 2021; Arpagaus, 2019). Spray drying is a suitable method to produce LAB starter culture powder as it has high productivity, low production cost, and ease to be scaled up compared to other methods such as freeze drying (Huang et al., 2017).

The principle behind spray drying is exposing the feed in a fluid state to a high-temperature gaseous drying medium (Cal & Sollohub, 2010). Inlet temperature in spray drying refers to the temperature of the gaseous drying medium which contacts with the incoming feed. Therefore, it directly affects the drying ability of the spray drying process (removed solvent per unit time) (Cal & Sollohub, 2010). Thus, the spray drying process may cause critical damage to the cells due to heat, dehydration (thermal shock), and oxidation stresses. Furthermore, these stresses may lead to the

alteration of metabolic activities due to cell wall, cell membrane, DNA, and ribosome damage, which becomes more fatal as inlet temperature increases (Peralta et al., 2017; Shokri et al., 2015).

Due to that, encapsulation is required to protect the cells from the stresses. Encapsulation refers to the coating of a substance using another substance that acts as a 'shell'. Encapsulation materials used for food-related production should be of food grade. Gum arabic, a biopolymer, is one of the most common gums used for encapsulation due to its emulsification properties, leading to emulsion stability (Mohammed et al., 2020). On the other hand, whey protein has been used as encapsulation material due to its beneficial gel properties (Sun et al., 2020). A study conducted by Sun et al. (2020), proved that the viability of spray-dried *Lactobacillus plantarum* A17 was improved due to the utilization of whey protein as encapsulation material in the spray drying. Furthermore, whey protein is known for its contribution towards the intermolecular cross-links with other biopolymers (Puttarat et al., 2021). In addition, protein and gum were a prevalent combination for encapsulation material, according to Mohammed et al. (2020). Therefore, gum arabic and whey protein will be used in this study. Spray drying on *P. acidilactici* has not been well studied, especially in analyzing the effect of different inlet temperatures. A study conducted by Noguerol et al. (2021) produced an antimicrobial powder of *P. acidilactici*, and thus was more focused on the antimicrobial activity of *P. acidilactici*. Therefore, the starter culture properties of spray-dried *P. acidilactici* have not been analyzed.

III. MATERIALS & METHODS

3.1. Time, Location, and Design of the Study

The research was conducted from September 2022 until December 2022 in the microbiology laboratory as well as the pilot plant of Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The experimental design of this research is shown in Figure 1 and described further in the next section.

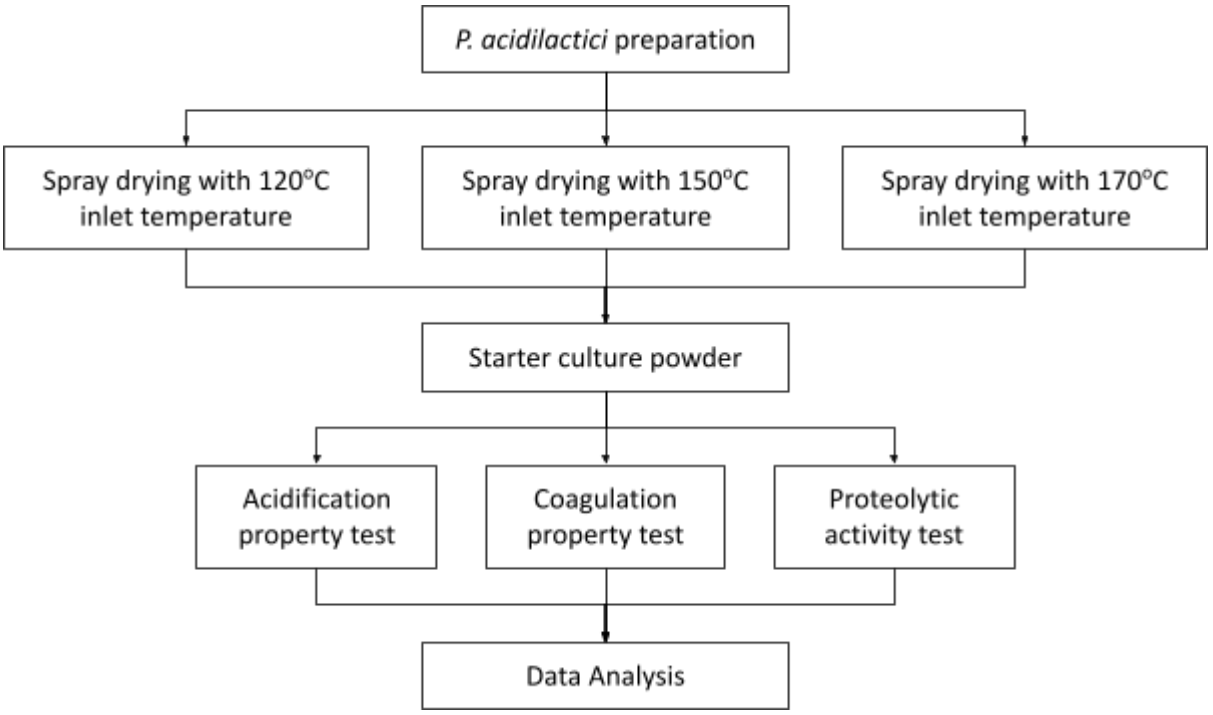

Figure 1. Experimental Design of the research.

3.2. Sample Preparation

P. acidilactici was obtained from i3L culture collection. The culture was initially activated by growing it in de Man Rogosa Sharpe (MRS) broth at 30°C for 18 h. Upon incubation, the culture was subcultured onto MRS agar, followed by another incubation at 30°C for 48 h. The agar was then stored in the fridge (4°C) to be used as working stock. Prior to the starter culture properties tests, the culture was reactivated in MRS broth at 30°C for 18 h.

3.3. Spray Drying

Prior to the spray drying process, the overnight culture was harvested through centrifugation at 25°C with 3500 rpm for 15 minutes. The cells were then washed twice using 15 mL of 0.9% saline solution and followed by concentrating the cells to log 7 CFU/mL by resuspending it in 10 mL of 0.9%

saline solution. Finally, the concentrated cells were transferred into a feed solution which consisted of water and 20% (w/v) encapsulation material (gum arabic and whey protein, ratio 1:1), and homogenized using a hand mixer for 5 minutes.

Spray drying was performed using a pilot-scale spray dryer (LPG 5, Changzhou Huaihai Drying Equipment Co., Ltd., Changzhou, China). The feed solution containing the *P. acidilactici* cells was fed into the spray dryer chamber at a constant flow rate of 15 rpm using a peristaltic pump (BT1005 basic variable-speed peristaltic pump, lead fluid). The spray drying was run in three different inlet temperatures: 120^{o} ± 2^{o}C, 150^{o} ± 4^{o}C, and 170^{o} ± 5^{o}C. However, the other parameters remained constant: atomization at 250 Hz, fan speed at 45 Hz, and air hammer within 1 second every 20 seconds. The resulting starter culture powders were moved into plastic zipper bags inside aluminium foil bags and stored in the fridge (4^{o}C).

3.4. Starter Culture Properties Characterization

3.4.1. Rehydration

Prior to the starter culture properties tests, samples were initially rehydrated. Rehydration was performed by dissolving 1 gram of each spray-dried *P. acidilactici* powder into 9 mL of 0.9% saline solution. Afterward, the powders were dissolved by mixing them using a sterile disposable inoculation loop.

3.4.2. Coagulation and Acidification

Coagulation and acidification tests were performed in the same sample, in which 300 µL of the rehydrated starter culture powders was inoculated into 30 mL of milk, followed by incubation at 30^{o}C for 24 h. The acidification property was observed by measuring the milk's pH using a pH meter. The same milk samples were then centrifuged at 4000 rpm for 10 minutes to separate between the curd and the supernatant (Cellesi et al., 2019; Wardani et al., 2017). The supernatant was removed for Lowry assay and the curd was weighed and calculated using Equation 1.

Equation 1:

$$Yield = \frac{curd\ weight}{milk\ weight} x100$$

The coagulation process resulted in the aggregation of insoluble casein. Thus, as supporting data, the Lowry assay was performed to quantify the remaining soluble protein in the supernatant. Bovine serum albumin (BSA) with a concentration of 1 mg/mL was initially prepared as control, followed by 2-fold dilution until the concentration reached 0.015625 mg/mL. Lowry assay was then performed to create the standard curve. Lowry assay was performed by adding 400 µL of Lowry's

solution, followed by vortexing and leaving it in the dark for 20 minutes. Afterward, 100 µL of Folin reagent was added, followed by vortexing and leaving it in the dark for 30 minutes. Absorbance at 750 nm was measured by Microplate Reader - The Infinite M200® NanoQuant by initially transferring the mixture to a 96-well plate (Hsieh, n.d.; Waterborg, 2009). A standard curve was then created. Lowry assay was then performed on the supernatant with the same protocol as for the standard curve.

3.4.3. Proteolytic

Skim milk agar was used to test the proteolytic activity of spray-dried *P. acidilactici*. Ten µL of the rehydrated starter culture powders were inoculated onto the skim milk agar, followed by 24 h incubation at 30°C. The skim milk agar had a white colour, in which after the incubation, a clear zone was formed as a result of proteolytic activity (Hamdani et al., 2019; Prescott & Harley, 2002). The diameter of clear zones and the colonies were measured, and the proteolytic index ratio (PIR) was calculated using Equation 2.

Equation 2:

$$PIR = \frac{diameter\ of\ clear\ zone - diameter\ of\ colony}{diameter\ of\ colony}$$

3.4.4. Viability

Miles and Misra method was implemented for the viable cell counting to ensure that the amount of rehydrated powder used in all tests were in the same concentration. Serial dilution was performed by initially preparing 900 µL of 0.9% saline solution on centrifuge tubes as the medium. Afterward, 100 µL of the rehydrated starter culture powders were transferred into the first dilution tube, followed by the 10-fold serial dilution up to 10^6 dilution factor. Afterward, 10 µL of each dilution factor was inoculated on MRS agar in triplicate, followed by incubation at 30°C for 48 h (Miles, Misra, & Irwin, 1938). The dilution factor consisting of 3-30 colonies of spray-dried *P. acidilactici* was used for the calculation on Equation 3 to calculate the viability.

Equation 3:

$$Log\ CFU/mL = log10(\frac{number\ of\ colonies * dilution\ factor}{0.01mL})$$

3.5. Data Analysis

All tests performed in the research were conducted in three biological replicates (n=3) with three technical replicates on each. One-way ANOVA (Analysis Of Variance) and Tukey post hoc tests were used to statistically analyze the results in GraphPad Prism 8.

IV. RESULTS AND DISCUSSION

4.1. Coagulation and Acidification Activities

As *P. acidilactici* has been utilized in numerous fermented products, it is important for the spray-dried cells to maintain their starter culture properties, including coagulation and acidification (Irmler et al., 2013; Kaya, & Şimşek, 2020; Olajugbagbe, Elugbadebo, & Omafuvbe, 2020). The properties were tested in milk as the activities are easier to observe (Akabanda et al., 2014; Li et al., 2022; Popović et al., 2020). Table 1 shows the results, including curd yield (%) obtained after isolating the curd through centrifuge, protein concentration (mg/mL) obtained from performing Lowry assay on the supernatant, and the final pH of milk after 24 h incubation with three different spray-dried *P. acidilactici* samples. All spray-dried samples showed coagulation activities, indicated by the formation of curds after the incubation. Centrifuging the incubated milk was intended to separate the curd from the supernatant (Figure 2) (Cellesi et al., 2019; Tavakoli, Najafi, & Mohebbi, 2019). Therefore, the curd yield from each sample was measured. The curd yields were not significantly different among the samples (P>0.05): 120°C (42.16 ± 1.41%), 150°C (42.28 ± 0.44%), and 170°C (42.28 ± 0.37%).

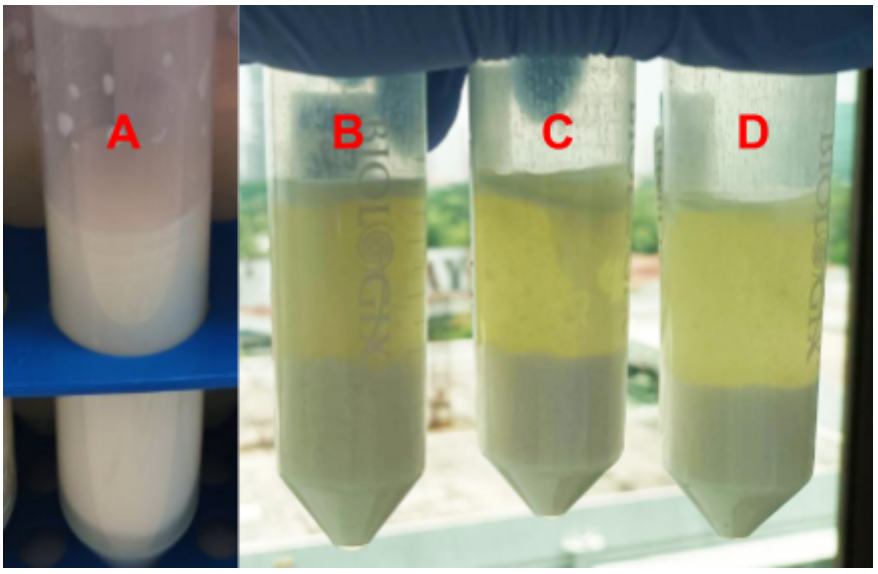

Figure 2. Separation of curd and supernatant from (A) uninoculated milk, (B) 120°C, (C) 150°C, and (D) 170°C spray-dried samples.

The coagulation activity was further explained by quantifying the remaining protein in the supernatant using the Lowry assay. Lowry method is a colorimetric assay which works by forming protein and copper complexes, followed by the addition of Folin reagent for the reduction reaction. This results in a color that is detected by the absorbance in 750 nm (Lu et al., 2010; Redmile-Gordon

et al., 2013). During coagulation, insoluble casein micelles are aggregated from the milk solution. Thus, Lowry assay was performed on the supernatant obtained from the centrifuged milk samples. The supernatant itself contained whey protein as well as the remaining soluble caseins (Cellesi et al., 2019; Chen et al., 2021; Dalgeish & Law, 1988). Prior to the assay, a standard curve was made using bovine serum albumin (BSA) with a concentration of 1 mg/mL, followed by two-fold serial dilution until 0.015625 mg/mL. As a result, a standard curve with R^2 value of 0.9976 (Figure 3) was obtained along with equation 4.

Equation 4:

$$y = 0.2405x + 0.0083$$

The equation was used to calculate the protein concentrations of samples. Based on the Lowry assay, uninoculated milk contained protein concentration of 29.48 ± 0.62 mg/mL. The concentration dropped significantly (P<0.0001) on all inoculated milks, with 150°C (3.90 ± 0.19) having the lowest protein concentration, followed by 120°C (4.34 ± 0.40) and 170°C (4.49 ± 0.37). Nevertheless, the final protein concentration of three different spray-dried samples did not differ significantly (Table 1). The results support the curd yield data, in which uninoculated milk had a much larger protein concentration, and the concentration dropped significantly compared to the milks inoculated with spray-dried samples.

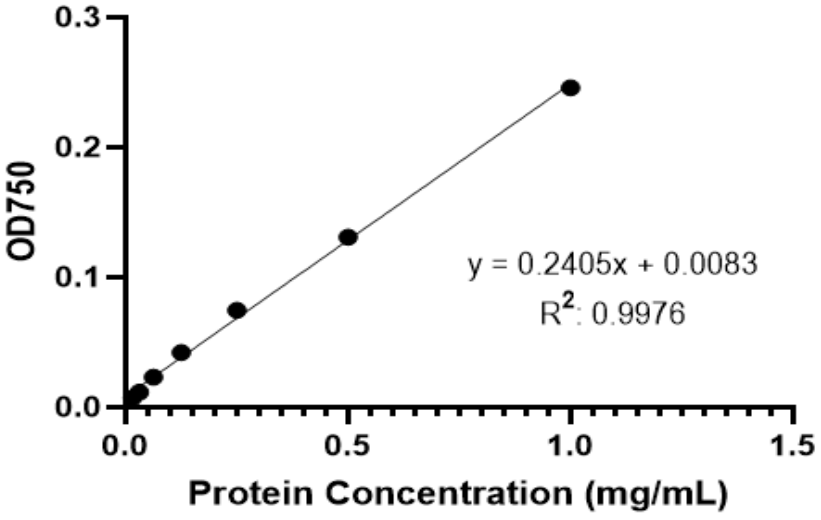

Figure 3. Standard curve of Lowry assay.

As a lactic acid bacteria, *P. acidilactici* has been known for its ability to produce organic acids such as lactic acid, which may acidify the environment. This property is commonly utilized in producing fermented foods as it may generate unique acid flavor and prolong the food shelf-life by inhibiting the growth of microbes that may cause food spoilage (Barbut, 2017; Csutak & Sarbu, 2018). To observe the acidification property of spray-dried *P. acidilactici*, both the initial and final

milk pH were measured. The initial milk had a pH of 6.65 ± 0.02. Meanwhile, after the milk was inoculated and incubated for 24 h with the spray-dried *P. acidilactici*, the pH dropped significantly. The lowest pH was achieved by the addition of 150°C (5.38 ± 0.09) spray-dried powder, followed by 120°C (5.43 ± 0.06) and 170°C (5.49 ± 0.08) (Table 1). However, statistical analysis showed that there were no significant differences between the final milk pH of each inoculated sample. Both results from coagulation and acidification tests indicated that spray-dried *P. acidilactici* still possesses the properties. In addition, the result was aligned with Boubellouta, Galtier, & Dufour (2011), in which milk coagulation can be achieved in the pH range of 5.5 to 5.0.

Table 1. Coagulation and acidification activities of different spray-dried *P. acidilactici* samples.

Sample	Curd Yield (%)	Protein concentration (mg/mL)	pH
Uninoculated milk	-	29.48 ± 0.62[a]	6.65 ± 0.02[a]
120°C	42.16 ± 1.41[a]	4.34 ± 0.40[b]	5.43 ± 0.06[b]
150°C	42.28 ± 0.44[a]	3.90 ± 0.19[b]	5.38 ± 0.09[b]
170°C	42.28 ± 0.37[a]	4.49 ± 0.37[b]	5.49 ± 0.08[b]

All data are the mean (n=3) ± standard deviation of three replicate samples. Means in the same column with different superscript letters indicates significant difference (P<0.0001) at ANOVA and Tukey's post hoc test, comparing all data in the same column.

4.2. Proteolytic and Viability

Growing bacteria on skim milk agar is one of the most common ways to observe the proteolytic activity of the microorganism, which has been implemented in numerous studies (Sepahy & Jabalameli, 2011; Fulzele et al., 2011; Marathe et al., 2018). The proteolytic index ratio (PIR) (Equation 2) was used in this study to qualitatively examine the proteolytic activity of the spray-dried *P. acidilactici* samples (Hamdani et al., 2019). Proteolytic activity can be observed by the presence of clear zones around the colonies grown on skim milk agar due to the hydrolysis of casein (Hakim et al., 2018).

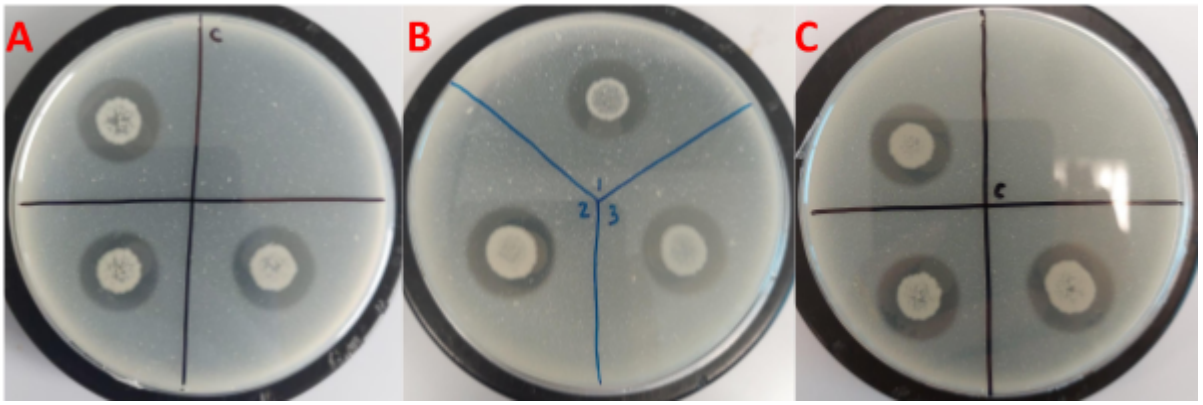

Figure 4. Proteolytic activity observed on skim milk agar: (A) 120°C, (B) 150°C, and (C) 170°C.

In this study, all of the spray-dried *P. acidilactici* samples showed positive results from the proteolytic test, which indicated that they exhibited proteolytic activity (Figure 4). The highest PIR value was generated from the 170°C (0.67 ± 0.03), followed by 150°C (0.60 ± 0.08) and 120°C (0.57 ± 0.03) (Table 2). However, the difference in the value did not show any significant difference. Prior to the starter culture properties test results, the viability of all the spray-dried samples were tested and the viability were not significantly different (P>0.05) (log ~6.9 CFU/mL) (Table 2). Therefore, the values on each test were comparable with each other.

Table 2. Proteolytic activity and viability of different spray-dried *P. acidilactici* samples.

Inlet Temperature (°C)	**Proteolytic Index Ratio (PIR)**	**Viability (Log CFU/mL)**
120	0.57 ± 0.03[a]	6.88 ± 0.03[a]
150	0.60 ± 0.08[a]	6.95 ± 0.03[a]
170	0.67 ± 0.03[a]	6.92 ± 0.05[a]

All data are the mean (n=3) ± standard deviation of three replicate samples. Means in the same column with similar superscript letters indicates no significant difference (P>0.05) at ANOVA and Tukey's post hoc test, comparing all data in the same column.

V. CONCLUSION

Three different inlet temperatures in spray drying were used to produce *P. acidilactici* starter culture powders for further analysis related to its effect towards the starter culture properties, including milk coagulation, acidification, and proteolytic properties. All tests showed positive results, in which all spray-dried *P. acidilactici* samples still retained the properties. The study found that different inlet temperatures do not affect the starter culture properties of spray-dried samples. Therefore, this finding can be used as a consideration for producing a commercial *P. acidilactici* starter culture powder in the future. Related to the inlet temperature, a specific temperature should be chosen based on the previous study in order to produce starter culture powder with the targeted yield, moisture content, and water activity. For future studies, it is recommended to investigate further other starter culture properties of spray-dried *P. acidilactici*, such as its carbohydrate metabolism and lipolytic activity. Analytical Profile Index (API) kit can be used to test the carbohydrate metabolism of spray-dried *P. acidilactici*. Furthermore, the lactic acid and pediocin production, as well as the safety assessment of the starter culture should be analyzed for future development.

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APPENDICES

Appendix 1. One-way ANOVA and Tukey post hoc test of curd yield from coagulation activity.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	4014	3	1338	F (3, 8) = 2315	P<0.0001
Residuals (within columns)	4.623	8	0.5779		
Total	4019	11			

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Uninoculated milk vs. 120°C	-42.16	-44.15 to -40.17	Yes	****	<0.0001
Uninoculated milk vs. 150°C	-42.28	-44.26 to -40.29	Yes	****	<0.0001
Uninoculated milk vs. 170°C	-42.28	-44.26 to -40.29	Yes	****	<0.0001
120°C vs. 150°C	-0.1179	-2.106 to 1.870	No	ns	0.9974
120°C vs. 170°C	-0.1175	-2.105 to 1.870	No	ns	0.9974
150°C vs. 170°C	0.0004667	-1.987 to 1.988	No	ns	>0.9999

Appendix 2. One-way ANOVA and Tukey post hoc test of protein concentration from coagulation activity.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1434	3	477.8	F (3, 8) = 2656	P<0.0001
Residuals (within columns)	1.439	8	0.1799		
Total	1435	11			

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Uninoculated milk vs. 120°C	25.14	24.04 to 26.25	Yes	****	<0.0001

Uninoculated milk vs. 150°C	25.58	24.47 to 26.69	Yes	****	<0.0001
Uninoculated milk vs. 170°C	24.99	23.88 to 26.10	Yes	****	<0.0001
120°C vs. 150°C	0.4333	-0.6757 to 1.542	No	ns	0.6149
120°C vs. 170°C	-0.1567	-1.266 to 0.9524	No	ns	0.9673
150°C vs. 170°C	0.5900	-1.699 to 0.5191	No	ns	0.3814

Appendix 3. One-way ANOVA and Tukey post hoc test of acidification activity.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	3.371	3	1.124	F (3, 8) = 243.6	P<0.0001
Residuals (within columns)	0.03690	8	0.004613		
Total	3.408	11			

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
Uninoculated milk vs. 120°C	1.225	1.048 to 1.403	Yes	****	<0.0001
Uninoculated milk vs. 150°C	1.270	1.093 to 1.448	Yes	****	<0.0001
Uninoculated milk vs. 170°C	1.168	0.9906 to 1.346	Yes	****	<0.0001
120°C vs. 150°C	0.04520	-0.1324 to 0.2228	No	ns	0.8458
120°C vs. 170°C	-0.05700	-0.2346 to 0.1206	No	ns	0.7389
150°C vs. 170°C	-0.1022	-0.2798 to 0.07538	No	ns	0.3222

Appendix 4. One-way ANOVA and Tukey post hoc test of proteolytic activity.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.01452	2	0.007262	F (2, 6) = 2.583	P=0.1551
Residuals (within columns)	0.01687	6	0.002811		
Total	0.03139	8			

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
120°C vs. 150°C	-0.02480	-0.1576 to 0.1080	No	ns	0.8390
120°C vs. 170°C	-0.09487	-0.2277 to 0.03797	No	ns	0.1514
150°C vs. 170°C	-0.07007	-0.2029 to 0.06277	No	ns	0.3091

Appendix 5. One-way ANOVA and Tukey post hoc test of viability.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	0.008089	2	0.004044	F (2, 6) = 2.935	P=0.1291
Residuals (within columns)	0.008267	6	0.001378		
Total	0.01636	8			

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
120°C vs. 150°C	-0.07333	-0.1663 to 0.01966	No	ns	0.1131
120°C vs. 170°C	-0.04000	-0.1330 to 0.05299	No	ns	0.4358
150°C vs. 170°C	0.03333	-0.05966 to 0.1263	No	ns	0.5484