The effect of pH on bacterial growth and histamine formation by *Klebsiella pneumoniae* CK02 and *Raoultella ornithinolytica* TN01

R Alya’ainun, E Y Fathoni and I D Puspita*

Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada, Jl. Flora A4, Bulaksumur, Yogyakarta, Indonesia

*Corresponding author e-mail: indun_dp@ugm.ac.id

Abstract. The present work describes the effect of pH on the growth rate and histamine formation by *Klebsiella pneumoniae* CK02 and *Raoultella ornithinolytica* TN01. Bacteria were inoculated on Tuna Fish Infusion Broth media with pH 5, 6, 7, 8 at 30°C for 6 hours. Sampling was conducted at 0, 3, and 6 hours to observe the bacteria number and calculate the histamine content formed in the medium. The number of bacteria was calculated using the Total Plate Count method, and the histamine content was analyzed using Thin Layer Chromatography with a combination of ImageJ software. Growth data and incubation time were plotted in the DMFit program to obtain growth rates. The effect of pH on growth rate and histamine formation was analyzed by ANOVA test and Duncan Multiple Range Test. The results revealed that pH affects the growth rate and histamine formation of *K. pneumoniae* CK02 and *R. ornithinolytica* TN01. The optimal growth rate of *K. pneumoniae* CK02 was in the range of 6-8 (0.304-0.380 log CFU/h), with the highest histamine formation ability at pH 7 (824 ppm). *R. ornithinolytica* TN01 had an optimal growth rate at pH 6-7 (0.480-0.508 log CFU/ml), with optimal ability to produce histamine at pH 6-8 (620-1,077.5 ppm). At pH 5, the growth rate and the ability of histamine formation by *K. pneumoniae* CK02 and *R. ornithinolytica* TN01 were inhibited.

1. Introduction

Tuna, mackerel tuna, and skipjack tuna are the most consumed fish (16.45%) based on national household preferences in 2016 [1]. Furthermore, this group of fish has a high protein content and other nutritious compounds which are beneficial for health. The Coastal Fishing Port of Sadeng is one of the ports in Yogyakarta that lands fish, including tuna, mackerel tuna, and skipjack tuna. The volume of skipjack tuna production in 2015 at Sadeng Port was 1,004 tons [2], while the mackerel tuna reached 505 tons in 2017 [3]. However, fish handling at Sadeng Port is not optimal for maintaining fish quality due to substantial temperature fluctuations and long fish handling times. Unloading skipjack tuna at Sadeng Port on a 15 GT vessel with an average catch of 4,135 kg took an average of 110 minutes and experienced an average temperature fluctuation of 7.1°C [4]. In the unloading of mackerel tuna on the >40 GT vessel, temperature fluctuations occurred ranging from 0.6-17.77°C with handling times ranging from 68-209 minutes, followed by a change in pH from 6.1 to 5.8 [5].
Tuna, mackerel tuna, and skipjack tuna are included in the Scombridae family which has a relatively high in the content of free histidine. Free histidine induces histidine decarboxylase-producing bacteria to produce histamine [6]. High levels of histamine in food potentially cause food poisoning. The increase in histamine in fish is influenced by the presence and abundance of free histidine, the presence of microorganisms, pH conditions, and temperatures that allow bacteria to grow and produce histidine decarboxylase (HDC) enzymes during processing and storage [7].

The effect of pH on histamine formation is based on the survival mechanism of bacteria in maintaining their growth in an acidic environment [8]. Bacteria use HDC to form histamine (an alkaline compound) to increase the environment's acidity level (pH) reaching the optimum pH for growth [9]. In acidic conditions, the induction of HDC gene expression occurs [10], meanwhile the HDC gene works slowly at alkaline pH resulting in HDC activity decreased at alkaline condition [11]. However, in some certain low acidity levels, the bacterial growth could be inhibited [12]. It indicates that changes in pH could affect the formation of histamine as well as bacterial growth.

The pH changes in fish can occur during handling or processing. Changes in fish pH during handling co-occur with quality deterioration. pH is one indicator to determine the level of fish freshness. Live fish have a pH of 7, but after death (pre-rigor), the remaining glycogen is broken down into pyruvic acid and then into lactic acid. The build-up of lactic acid causes the pH to drop. Furthermore, at the rigor mortis stage, the pH continued to decrease to 6.2–6.6 [13]. After that, the fish enter the post-rigor phase, where the pH increases until it becomes rotten and fish pH reaches 7.7-8.

Various species of histamine-forming bacteria (HFB) can be isolated from fish. The HFB found in fish are mostly from the Enterobacteriaceae class, such as Enterobacter aerogenes, Roulletella planticola, and Morganella morganii [14]. The three bacteria are high histamine-producing bacteria (>1000 ppm). Another study reported a total of 44 HFBs that have been identified from skipjack tuna and mackerel tuna [15]. Klebsiella spp. is one of 21 isolates identified to produce histamine in large quantities (>1,000 ppm) together with M. morganii, H. alvei, and Proteus spp. The Laboratory of Quality Control and Safety of Fishery Products UGM has a collection of Klebsiella pneumoniae CK02 and Raoultella ornithinolytica TN01 isolated from skipjack tuna and mackerel tuna. K. pneumoniae CK02 and R. ornithinolytica TN01 produced histamine of 1,384 ppm and 935 ppm, respectively [16][17].

This study aimed to determine the effect of pH on the growth rate and histamine formation by K. pneumoniae CK02 and R. ornithinolytica TN01. This study is expected to explain the relationship between pH with growth and histamine formation by HFB. Thus, it can be used as initial information to prevent the formation of histamine in fish during handling and processing.

2. Materials and Methods

2.1. Medium preparation

The medium consisted of Tryptic Soy Agar (TSA) (Oxoid), Tryptic Soy Broth (TSB) (Oxoid), and Tuna Fish Infusion Broth (TFIB). TSA was used as a medium for bacterial growth, while TSB was used in the preparation stage of bacterial culture. TFIB was used in testing the growth of histamine-producing bacteria and their ability to produce histamine. The preparation of the TFIB medium referred to Chen et al. [18]. The tuna fillet meat was washed and then homogenized with aquadest twice the weight of the fish meat and then boiled at 100°C for 1 hour. The cooled fish stew was then filtered with Whatman #1 filter paper and add 1% glucose. Furthermore, the initial TFIB medium, which had an initial pH of 6, was adjusted using 1 N lactic acid for acidic conditions and 1 N NaOH for alkaline conditions. Then, the TFIB medium whose pH had been adjusted to 4, 5, 6, 7, and 8 were sterilized at 121°C for 15 minutes.

2.2. Measurement of bacterial growth and histamine formation

A total of 1 inoculating loop (ose) of K. pneumoniae CK02 and R. ornithinolytica TN01 from TSA slant medium was grown in 10 ml TFIB with various pH (5, 6, 7, and 8) then incubated at 30°C. All the treatment were carried out in two replication. Observations were made at 0, 3, and 6 hours. During the
observation, the number of bacteria and the concentration of histamine formed in the medium were measured.

Bacterial growth was analyzed using the modified TPC method [19]. Modifications were made by changing the number of samples grown in TSA from 0.1 ml to 20 µL. Incubation was carried out at 37°C for 24 hours. The measurement of the amount of histamine was carried out by the TLC method [20]. A total of 50 µL samples were centrifuged for 10 minutes at 6,000 rpm. A total of 0.5 µL of supernatant was applied to the TLC plate (Silica Gel 60 F254, Merck). The compound's separation was carried out in a chamber with a mobile phase (solvent) of Methanol and Ammonia with a ratio of 20:1 (v/v). The plate was inserted into the chamber containing the solvent and waited until the solvent reached the upper limit. The plate was then removed from the chamber, dried using a hairdryer, then sprayed with ninhydrin solution (300 mg ninhydrin in 100 ml n-butanol, added with 3 ml glacial acetic acid), and then dried using a hairdryer for spot visualization. Determination of histamine and histidine levels was carried out using densitometric analysis based on spot area using ImageJ software [21]. The standards marked on the TLC plate were 300-1,300 ppm for histamine and 200-1,000 ppm for histidine.

The histamine and histidine standard spot areas from the ImageJ software were made a linear graph using the Microsoft Excel program, and the linear regression equation y=ax+b was obtained.

2.3. Data analysis
Growth data (log CFU/ml) at various pH and incubation times were plotted with a Microsoft Excel program to obtain bacterial growth curves. Prediction of bacterial growth rate (µ) was analyzed based on the growth model of Baranyi and Roberts [22] using the DMFit program. The DMFit program is an Excel add-in used to fit a growth curve based on a quadratic equation.

The effect of pH on bacterial growth rate (µ) and histamine formation was analyzed using the ANOVA test with the SPSS program. The experiment used a completely randomized design with two replications. If the data analysis has a significant effect, it was proceed with the Duncan Multiple Range Test (DMRT). DMRT aimed to determine the difference between each treatment.

3. Results and Discussion

3.1. Effect of pH on the growth of K. pneumoniae CK02 and R. ornithinolytica TN01
Table 1 shows that K. pneumoniae CK02 was able to grow at pH 6, 7, and 8. The number of K. pneumoniae CK02 cells increased by 1.831, 2.283, and 2.486 log CFU/mL at pH 6, 7, and 8, respectively, after being incubated for 6 hours at 30°C. A similar trend was also seen in the growth of R. ornithinolytica TN01. The number of cells at pH 6, 7, and 8 increased by 3.048, 2.882, and 2.196 log CFU/ml after 6 hours incubation. While at pH 5, both bacteria showed a decrease in the number of cells. The ANOVA analysis showed an effect of pH (p<0.05) on the number of K. pneumoniae CK02 and R. ornithinolytica TN01 at 6 hours of incubation. DMRT test showed that the number of bacteria in the pH 5 was significantly different from the other pH, while the number of bacteria in pH 6, 7, and 8 was not significantly different.
Table 1. The growth rate of *K. pneumoniae* CK02 and *R. ornithinolytica* TN01 in TFIB with various pH treatments.

| Isolate                  | pH | Cell number (log CFU/mL) | µ (log CFU/h)¹) |
|--------------------------|----|--------------------------|----------------|
|                          | 0  | 3                        | 6              |
| *K. pneumoniae* CK02     | 5  | 5.635                    | 5.605          |
|                          | 6  | 5.115                    | 6.007          |
|                          | 7  | 5.678                    | 6.447          |
|                          | 8  | 4.659                    | 5.375          |
| *R. ornithinolytica* TN01| 5  | 3.680                    | 3.394          |
|                          | 6  | 3.429                    | 4.578          |
|                          | 7  | 3.861                    | 4.400          |
|                          | 8  | 4.275                    | 5.325          |

³⁴ different letters in the same column indicate a significant difference (P<0.05) for *K. pneumoniae* CK02

²³ different letters in the same column indicate a significant difference (P<0.05) for *R. ornithinolytica* TN01

¹) Bacterial cultures were incubated at 30°C for 6 hours

It is in line with the results of ANOVA analysis on the growth rate of bacteria, which showed an effect of pH (p<0.05) on the growth rate of *K. pneumoniae* CK02 and *R. ornithinolytica* TN01. DMRT test showed that the pH range of 6-8 was different from pH 5 in the growth rate of *K. pneumoniae* CK02. Meanwhile, in *R. ornithinolytica* TN01, pH 6 and 7 was different from pH 5 and the growth rate at pH 8 was lower when compared to pH 6 and 7. Tsuji et al. reported that *K. pneumoniae* has a high growth rate and a short generation time in the pH range of 6–8 [23]. Tantasuttikul & Mahakarnchanakul also reported that *R. ornithinolytica* grown on TSB medium showed the highest growth value after 6 hours of incubation at pH 6 and 7, while at lower pH (4-5) and higher pH (8-10) the number of cells decreased [24]. It indicates that the optimal pH range for the growth of *K. pneumoniae* CK02 is broader (pH 6-8) than that of *R. ornithinolytica* TN01 (pH 6-7).

At pH 5, the growth rate of the two bacteria showed a negative value indicating cell death. Bacteria have a mechanism to maintain a constant/optimum pH in their cells when there is a decrease in the environment’s pH. When the pH is lowered from the optimum pH, protons present in high numbers in the medium will enter the cell's cytoplasm. Furthermore, these protons must be removed from the cell to prevent acidification and denaturation of cell components. This proton removal process requires energy, the amount of which depends on the high and low pH. As a result, the energy for cell growth is reduced, and the growth may stop altogether. Highly acidic conditions cause damage to the intracellular components of bacteria so that they can cause bacterial cell death [25].

3.2. Effect of pH on histamine formation by *K. pneumoniae* CK02 and *R. ornithinolytica* TN01

Histamine is formed from the decarboxylation of histidine in the TFIB medium. The amount of initial histidine in the TFIB medium ranged from 432-1,661 ppm. Histidine levels at hour 0 were not the same for each treatment. Histidine levels in the TFIB medium tended to decrease along with the decrease in the pH value. It may be because histidine is degraded at low pH. Histidine has a base side-chain, namely imidazole, which is positively charged at neutral pH. Histidine will be protonated at neutral to alkaline pH. The protonated histidine residue will facilitate the enzyme-catalyzed reaction [26]. Along with the formation of histamine, histidine levels in the medium decreased. At the end of the observation (6 hour), there was still histidine remaining in the medium (426-1299 ppm) which indicated that not all histidine available in the medium was used in the decarboxylation reaction.
Table 2. Effect of pH on histamine formation by *K. pneumoniae* CK02 and *R. ornithinolytica* TN01 on TFIB medium after 6 hours of incubation at 30°C.

| Isolate                | pH | Replication 1 | Replication 2 | Average |
|------------------------|----|---------------|---------------|---------|
| *K. pneumoniae* CK02   | 5  | ND            | ND            | ND^d    |
|                        | 6  | 524           | 494           | 509^b   |
|                        | 7  | 649           | 666           | 824^a   |
|                        | 8  | 504           | 509           | 507^b   |
| *R. ornithinolytica* TN01 | 5  | ND            | ND            | ND^b    |
|                        | 6  | 580           | 1575          | 1,077.5^A |
|                        | 7  | 680           | 890           | 785^A   |
|                        | 8  | 555           | 685           | 620^A   |

ND = Not detectable; all the ND data was converted as 0 for the statistical analysis

^a-b different letters in the same column indicate a significant difference (P<0.05) for *K. pneumoniae* CK02

^A-C different letters in the same column indicate a significant difference (P<0.05) for *R. ornithinolytica* TN01

In this study, histamine levels were tested at 0, 3, and 6 hours. However, at 0 and 3 hours, histamine levels could not be detected. Histamine levels were only detected at pH 6, 7, and 8 at 6 hours of incubation. Histamine formation by *K. pneumoniae* CK02 and *R. ornithinolytica* TN01 at 6 hours of incubation in TFIB medium with various pH treatments ranging from 507-824 and 620-1,078 ppm, respectively. ANOVA test showed that the pH treatment affected the formation of histamine (p<0.05) by *K. pneumoniae* CK02 and *R. ornithinolytica* TN01. Based on the DMRT test, it was found that the histamine formation by *K. pneumoniae* CK02 at pH 7 was different from pH 5, 6, and 8. In *R. ornithinolytica* TN01, the histamine levels at pH 6, 7, and 8 were not different, but at pH 5 were different.

At pH 5 treatment, histamine levels were not detected in the medium. It follows Taylor & Woychik’s statement [27], who reported that histamine production by *Klebsiella pneumoniae* occurs very slowly at pH 5.3. Most production occurs between 12 and 24 hours. In addition, Dityanawarman et al. [16] reported that histamine production in the TFIB medium tends to be lower than in the TSBH medium. The HDC enzyme in *Raoultella planticola* bacteria works optimally at pH 6.5 with a pH range of 6–8, whereas its activity is low at pH 5 and decreases at pH 4.5 [28].

Based on this research, it can be concluded that the bacterial growth rate does not always correlate with the bacteria’s ability to produce histamine. It was shown by *K. pneumoniae* CK02 which had no different growth rates at pH 6, 7, and 8 but produced significantly higher histamine at pH 7. Similarly, *R. ornithinolytica* TN01 showed a significantly lower growth rate at pH 8 but produced histamine, which was not significantly different at pH 6, 7, and 8. It could be assumed that pH has different mechanisms in influencing bacterial growth and the performance of HDC enzymes. According to Wang et al. [8], bacteria can produce histamine specifically under acidic conditions because acidic pH increases gene expression and increases the formation of histamine, which is excreted from the cell. When bacterial cells are threatened in an acidic pH environment, cells will release large amounts of H+ by converting histidine to histamine and CO₂ as a survival strategy. Therefore, the bacteria can be freed from acid pressure. According to Diaz et al. [10], the increase in histamine production at acidic pH is caused by the induction of gene expression or structural changes in the HDC gene that can increase enzymatic decarboxylation activity. Further research to determine the mechanism of pH in influencing the physiology of histamine-forming bacterial cells and cell metabolism related to the formation of HDC is needed.

4. Conclusion

This study presented the effect of pH on growth and histamine formation ability in *K. pneumoniae* CK02 and *R. ornithinolytica* TN01. The optimal growth rate of *K. pneumoniae* CK02 ranges from pH 6-8 (0.304-0.380 log CFU/h), with the highest histamine formation ability at pH 7 (824 ppm). *R. ornithinolytica* TN01 has an optimal growth rate in the pH range of 6-7 (0.480-0.508 log CFU/ml), with
an optimal ability to produce histamine in a broader range of pH 6-8 (620–1,077.5 ppm). At pH 5, the growth rate and histamine formation ability of K. pneumoniae CK02 and R. ornithinolytica TN01 is inhibited. The application of low pHs, such as weak organic acids as a solvent of natural preservatives (such as chitosan and its derivatives), could be an alternative hurdle technology in inhibiting the growth of histamine-forming bacteria in fresh fish.

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