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EVALUATION ON METHODS FOR IDENTIFICATION OF VIBRIO SP.: KAWAKAWA (EUTHYNNUS AFFINIS) BRINING SHREDDED AND CHITOSAN ADDITION AS PRESERVATIVE

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Abstract

The kawakawa (Euthynnus affinis) brine salting that used to make shredded were preservative with salt. Salt addition has aim to inactivate of bacterial contamination. Obviously, bacteria is still capable of growing in kawakawa brine shredding Therefore, in this study chitosan was added as antibacterial in shredded processing. Vibrio sp., is one of common halophilic bacteria found in seafood. If this bacteria is consumed, it can cause serious problems in human health. The objective of this study was to evaluate the factors that affect in identifying Vibrio sp., on kawakawa brine shredding (C), shredded non-chitosan (FC) and shredded contain of chitosan (FC+). The methods evaluated were steps in enriching bacteria, culturing bacterial in selective media and analysing bacterial by API 20E kit. Enriching and incubation periods were needed by halophilic bacteria to adaptation in new environment. It was required to observe the bacteria characteristics' that would be isolated. Bacterial colonies were growth on Thiosulphate citrate bile salt sucrose (TCBS) were not Vibrio sp., but confirmed as Pseudomonas luteola and Proteus vulgaris based on API 20E analysis. It was showed that TCBS media had some advantages in identifying Vibrio sp. In conclusion, to get the best result in identifying bacteria, at least two or more methods were used to avoid misidentification.

Keywords: *Bacterial; Biochemical Reaction; Seafood; Preservative*

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INTRODUCTION

Preservation of tuna (*Euthynnus affinis*) using salt or so-called shredding technique, is one of the conventional techniques in the fish preservation process. The salt used is to replace the water molecules with the salt molecules in the fish flesh. This substitution causes a decrease in the water content in fish meat and of course it is directly proportional to the decrease in water activity (AW). This condition will be able to inhibit the growth of bacteria, because water is the main factor needed by bacteria to grow (Bonoco & Kurt Kaya, 2018). However, it is unfortunate that halophilic and thermophilic bacteria can become bacteria that contaminate processed seafood. Even though it has been treated with salt and high temperatures, due to the ability of these bacteria to live in an environment with high levels of salinity and temperature (Deib *et al.,* 2013; Azwai *et al.*, 2016; Sumitha *et al.,* 2018). Generally, some halophilic bacteria only require room temperature to grow which enters the mesophilic bacteria (Alfonzo *et al.,* 2017). This halophilic bacterial contamination can come from bacteria in the marine environment, in the fish itself, or the water and salt used in the shredding process. It can also come from the processing environment and the people who make the shredding (Sergelidis *et al.,* 2014). Therefore, it is necessary to do further processing in shredding. Shredding processing becomes another processed menu, one way to reduce bacterial contamination. Processing menus at 80°C to 180°C, such as frying and boiling or steaming, is known to kill bacteria (Yin *et al.,* 2014; De Jong *et al.,* 2012). In previous studies, it was explained that the use of preservatives in food processing can be added as an antibacterial and can extend shelf life (Sultana *et al.,* 2019; Das *et al.,* 2009; Niederer *et al.,* 2019). Some food preservatives such as sodium nitrite, sodium glutamate and chitosan are often added to food as preservatives. In recent years, chitosan is being studied for its ability to preserve food (Shanmugam *et al.,* 2016).

In this study, chitosan was used as a preservative in the manufacture of shredded shredding cob (*Euthynnus affinis*). The addition of chitosan aims to inhibit the growth of *Vibrio sp*. The reason of choosing *Vibrio sp*., it was seen from the characteristics that *Vibrio sp*., commonly found in raw seafood and processed seafood, is able to adapt to salinity and high temperature (Baker *et al.,* 2018). There are several methods of identification of bacteria such as the use of selective media, gram staining and

analytical kits (Vithanage *et al.,* 2014; Hikmawati *et al.,* 2019).

Each method has advantages and disadvantages, so identification errors are possible. However, they can also support each other in establishing identification. The combination of using several methods in the identification process can prevent or minimize human errors during identification (Giuliano *et al.,* 2019). In this study, an evaluation of two methods used in determining the identification of *Vibrio sp*. Evaluation is directed towards problems that arise during the identification process.

The method used was using selective media Thiosulphate citrate bile salt sucrose agar (TCBS) and Analytical Profile Index (API) 20E. Selective media were usually used for the identification of specific bacteria, where the content of substances in the media was adjusted to the needs of certain bacteria. In this study, TCBS was used as a selective medium for *Vibrio sp*. The results of growing bacteria will be compared with the identification results of API 20E. The objective of this study was to examine and to evaluate the factors that influence the failure of handling bacteria, during the process of planting *Vibrio sp*. in TCBS media and identification of *Vibrio sp*.

RESEARCH METHODS

This study was conducted from July to August 2019. Kawakawa used in making shredded were purchased at Badung Market, Bali. Shredding was made in the Kitchen laboratory, Nutrition Science Study Program, Dhyana Pura University, Bali. While the cultivation of bacteria and analysis of *Vibrio sp.,* in TCBS media was carried out at the Microbiology Laboratory, Faculty of Medicine, Udayana University, Bali. Analysis of bacterial samples using API 20E was carried out at NIKI Laboratory, Bali.

In this study, materials used were such as chitosan, kawa-kawa (*Euthynnus affinis*), Kawakawa brine shredding, *sodium chloride solution (NaCl), Tryptic Soy Broth (TSB) and Thiosulphate citrate bile salt sucrose agar (TCBS)*. The instruments used were incubator, petri dish, test tube, micropipette and Analytical Profile Index (API) 20E kit.

The samples tested were 3 samples, namely Kawakawa shredding as a negative control, Shredding which was processed into shredded without the addition of chitosan and Shredding which was made into shredded with the addition of 50 mg of chitosan.

Making samples of kawakawa brine shredding (*Euthynnus affinis*). kawakawa brine shredding were cleaned of bones, head and fish belly. The shredding was shredded

and weighed as much as 100 grams for each shredded sample. The sample was shredded with chitosan, then 50 mg of food grade chitosan was added into 100 g of shredded and allowed to stand for 5 minutes. Then the shredded was processed by the method of stir frying with spices and then deep frying in hot oil. The shredded that had been weighed as much as 100 grams were stored in a glass jar. Sample symbols for shredding without being processed into shredded (C), shredded without chitosan (FC-) and shredded with chitosan (FC+).

Bacterial cultivation on TCBS media was carried out by weighing 1 gram of each sample. Then diluted through a dilution rate of 10-1 to 10-4 in NaCl solution. Then two planting methods were carried out, namely 10 microliters were directly taken from the 10-4 dilution tube and planted on TCBS media with the spread method. The second method took 1 ml from a 10-4 dilution tube and put it into 9 ml of TSB solution as an enrichment. TSB media was incubated for 24 hours at 37°C. After 24 hours, 10 microliters were taken from the TSB media and implanted on the surface of the TCBS media using the spread method. Then incubated at 37°C for 24 hours. The growing bacteria were inoculated again to obtain pure colonies in new TCBS media by streak method by taking 1 colony of growing bacteria. The growing bacterial colonies were continued by observing the morphology with the naked eye and 1 colony was taken for analysis using the API 20E device. The results of both method will be compared whether the bacteria that grow in TCBS selective media are *Vibrio sp*., as the results of the API 20E analysis (Djaouda *et al.,* 2013; Wittriansyah *et al.,* 2019). The results obtained both based on observations of the morphology of the colonies growing on TCBS media and the results of the API 20E analysis, will be discussed descriptively based on the problems that arise during the study.

RESULTS AND DISCUSSION

Evaluation in Sample Enrichment

The results of the planting of the three samples were shredding cob (C), shredded without chitosan (FC-) and shredded with additional chitosan (FC+), which were taken directly from dilution of 10-4 NaCl solution into TCBS media, after being incubated for 24 hours at 37° C can be seen in Figure 1. The figure shows no bacterial colonies growing on the surface of the TCBS media. Incubation was extended to 72 hours, but still no bacterial growth.

It occurred since *Vibrio sp.,* which is classified as halophilic bacteria, grew very slowly compared to non-halophilic bacteria

or mesophilic bacteria in general. (Lee *et al.,* 2018; Tippayawong, 2016). Halophilic bacteria require not only the sodium (NaCl) component in order to grow (Arisandi *et al.,* 2017). However, halophilic bacteria also require other nutrients such as fish extract, milk, other protein sources and even complex nutrients such as yeast extract. Previous study in growing *Vibrio sp.*, before planting in TCBS, enriched in *alkaline peptone water* (APW) or *tryptic soy broth* (TSB). APW and TSB media can be used alone or added with NaCl. In the enrichment process, halophilic bacteria need about 18-24 hours at 37°C. This technique would give the halophilic bacteria an opportunity to adapt, so that they can grow (Youssef *et al.,* 2018; Fadel & El-Lamie, 2019). This explanation was able to answer why Vibrio sp., which was expected to grow on TCBS media in this study, did not grow. Where the first technique was the isolation process of *Vibrio sp*., planting was carried out directly into TCBS from a NaCl solution of 10^{-4} dilution, without going through an enrichment process. The NaCl solution contained few nutrients and did not give bacteria the opportunity to adapt to the new environment. In the study of Chakma *et al.,* (2018), it was only using Phosphate Buffer Saline (PBS) solution to isolate *Vibrio sp*., from shrimp samples. However, before being planted into TCBS, the suspension solution was incubated for 24 hours. Hikmawati *et al.,* (2019) were known to only use sterile aquadest solution when isolating *Vibrio sp*. from green mussels (*Perma viridis*) and directly planted on TCBS, then incubated for 48 hours. The difference with the isolation of *Vibrio sp.* in this study was Vibrio bacteria isolated from tuna that had been processed into shredding and shredded fish. *Vibrio sp*., belongs to the group of thermophilic bacteria that have enzymes that can still be active at high temperatures. Isolation of halophilic bacteria from fresh samples, not yet through a food processing process involving heating, cooling, and preservation will affect the adaptation time. Hence it can be concluded that the enrichment media and adaptation time are two important factors that influence the growth of halophilic bacteria.

On planting into TCBS media, the results of the enrichment of the three samples came from dilution of 10^{-4} NaCl solution into TSB media. After being incubated at 37°C for 24 hours. A total of 10 microliters were taken from TSB media and planted in TCBS media, and incubated for 24 hours at 37°C. In Figure 2. it shows TCBS media, the control sample (C) and shredded without chitosan (FC-) planting showed colonies growing. The

difference is the colonies in the control sample grew more fertile than the shredded samples without chitosan. Meanwhile, in the sample of shredded chitosan (FC+) no colonies grew. Based on these results, the addition of chitosan as a preservative has antibacterial properties (Nile *et al.,* 2020).

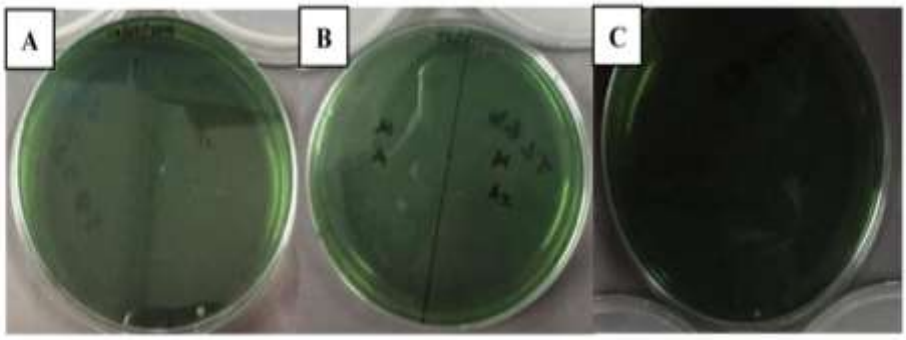

Figure 1.The results of the planting of the three samples from a 10^{-4} dilution in a solution of NaCl de Media TCBS. All three has no bacteria grew after incubation. A. Control (C); B. Shredded without chitosan (FC-); C. Shredded chitosan (FC+)

Evaluation of Isolation and Identification of *Vibrio sp*.

Colonies purified from the control sample (C) and shredded without chitosan (FC-) after observing the morphology, both had a yellow color. Only the bacterial colony size of the control sample (C) was larger and the growth was fast compared to the shredded sample without chitosan (FC-) which had small and few colony sizes (Figure 2.). Pramono *et al.,* (2015), explained that the process of traditional seafood, such as shredding, was still in its semi-cooked form but had not been able to reduce the contamination of *Vibrio sp*. *Vibrio sp*., is a thermophilic bacterium that is able to adapt to an environment of 45-90°C. Frying process with temperature ±180°C when the shredding was processed into shreds, is believed to be able to kill some bacteria (Golgolipour *et al.,* 2019). Seafood processing with temperatures above 70°C, is quite effective in inhibiting the development of pathogenic and non-pathogenic bacteria (De Jong *et al.,* 2012).

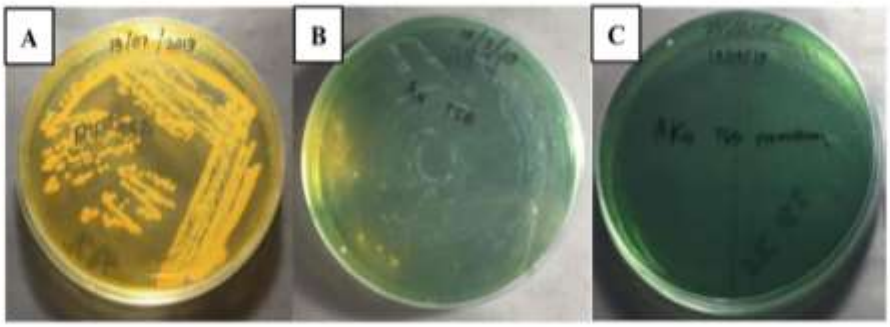

Figure 2. Colony morphology *Vibrio sp*. Was grown on TCBS media. A. Control (C); B. Shredded without chitosan (FC-); C. Shredded with chitosan (FC+)

To further confirmation whether the bacteria growing on the TCBS media were *Vibrio sp*., then bacterial analysis was continued with the API 20E device. The result was not identified as *Vibrio sp*., but *Pseudomonas luteola* and *Proteus vulgaris*, from shredded samples without chitosan (FC-) and control (C). This unexpected result can be explained in Shikongo-Nambabi's study (2012), that TCBS media is not always selective for *Vibrio sp.,* but can also grow other halophilic bacterial families such as *Pseudomonas sp*. and *Proteus sp.* Especially if the sample comes from marine fish and processed seafood. However, if it is not *Vibrio sp.*, the colonies that grow will be transparent and difficult to develop. It is due to TCBS media contains sucrose, where *Vibrio sp*. are able to ferment sucrose will be yellow, while those that are not, will be green. Yeung *et al*., (2016) reported the level of specification and sensitivity of TCBS to *Vibrio sp*., which is lower than that of *Chromogenic agar*. Another weakness of TCBS media is the inability to produce so that morphologically *Proteus* colonies can be distinguished from *Vibrio sp*. (Tippayawong, 2016; Cira *et al.,* 2012). Most of the halophilic bacteria isolated from marine animals belong to moderate halophilic bacteria that can live at salinity levels of 5-20%. The ability of each species of halophilic bacteria to produce different structures of extremozymes as a result of bacterial activity during adaptation to a high salinity environment. Chemical reactions can give different colony coloration in *Vibrio sp*. (Yeung & Thorsen, 2016; Cira *et al.,* 2012; Sebastião *et al.,* 2015; Drzewiecka, 2016; Murugan *et al.,* 2018). Several studies have been carried out to develop and modify selective media for *Vibrio sp*., using a mixture of pure seawater and pure salt in the form of 30 g/L marine coral reef aquarium salt or 27 g/L NaCl *(*Tagliavia *et al.,* 2019).

The Analytical Profile Index (API) 20E analysis tool was a biochemical analytical test. This device was capable of identifying bacteria to the level of strain determination. When compared with the use of selective media, the biochemical analysis of API 20E would be interpreted through software based on stored data. After finishing analysing the chemical reaction to the bacterial sample, the next step was scoring based on changes in chemical reactions based on the manual. The last stage was entering numerical values into the software for the identification process. The level of confidence was shown in the form of a percentage value (%). In addition to the percentage, it was also described as *good, very good, excellent, acceptable identification, exact, nearest identity to none* (Maina et al., 2014). In this study, the results of the API 20E analysis can be seen in Table 1. compared with the results of identification based on morphological observations. Basically the identification method of TCBS and API 20E used the same principle. The principle was by observing the ability of bacteria to break down the substrate during the metabolic process, which produced a product or metabolic product that was detected by a color changing. The difference is, API 20E had supporting data not only based on morphology, namely color, which can be false positive or negative. Therefore, in this study, the results of bacterial identification on TCBS selective media against growing colonies could be *Vibrio* sp., or possibly *Enterobacteriae sp.* or *Pseudomonas sp*. Identification of bacteria with API 20E confirmed *Pseudomonas luteola* (FC-) and *Proteus vulgaris* (C). Similar results were reported by Aruwa and Olatope (2015) for the identification of Bacillus sp., using the classical method, namely tests based on biochemical reactions and API 20E giving different results. Bacillus was isolated from food samples and analysed by classical methods confirmed as *B. licheniformis*. However the results of the analysis with API 20E confirmed as *B. substilis* (95.6%). In the end to establish the identification of a bacterium required 2 or 3 methods to provide the best confirmation. It was even better to develop or update the old methods to increase their specificity and sensitivity, so as to provide more accurate identification results.

Table 1. Results Identification of *Vibrio sp*. with TCBS media and analytics API

Isolate code	TCBS identification			API Identification (%ID)
	Bacteria	Colour	Growth	
C	*Vibrio alginolyticus*	Yellow	Fast	*Proteus vulgaris* (99.2%)
	V. cholerae	Yellow	Fast	
	V. furnissii	Yellow	Fast	
FC-	*V. fisheri*	Yellow	Weak	*Pseudomonas luteola* (98.1%)

C (control: Kawakawa shredding (Euthynnus affinis); FC- (shredded without chitosan)

CONCLUSION

The process of identifying bacteria used at least two methods to confirm the results and to observe the characteristics of the bacteria to be isolated. Weaknesses and strength of the chosen method was to avoid the bacterial identification errors. Identification of *Vibrio sp.,* in this study was not obtained in accordance with the purpose of using the selective media method, but it did not mean failed. Identification by analysis of API 20E, the use of chitosan as a preservative was able to become antibacterial in the process of shredded. Therefore there was no bacterial contamination of *Vibrio sp., Pseudomonas luteola and Proteus vulgaris*.

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