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The effect of inoculation of *Aspergillus oryzae* and *Rhizopus oligosporus* in molds fermentation on the characteristics of pigeon pea sauce (*Cajanus cajan* (L.) Millsp.)

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Abstract
This study aimed at examining the effect of *waru* leaves (*Hibiscus tiliaceus* L.) as a source of molds, *Apergillus oryzae,* and *Rhizopus oligosporus* in the process of solid-state fermentation (koji making) and salinity in the process of brine fermentation to yield *moromi* (*baceman* fermentation) towards the characteristics of pigeon pea sauce. This study used a factorial randomized block design (RBD) consisting of two factors. The first factor was the inoculum treatments, which included: (1) control: the process of making koji using *waru* leaves (*H. tiliaceus* L.); (2) the process of making koji using *Rhizopus oligosporus*; (3) the process of making koji using *Aspergillus oryzae*; and (4) the process of making koji using *R. oligosporus* and *A. oryzae*. The second factor was soaking in a saline solution with concentrations of 15%, 20%, 25%, and 30% (w/v) and the fermented pigeon peas were grouped twice according to the time of processing. The results showed that the mixed inoculum treatments and 20% salt content produced the best pigeon pea sauce characteristics. The characteristics of the pigeon pea sauce were as follows: water content 80.33%, protein content 4.04%, salt content 19.41%, pH 6.2, total acid 0.22%, lactic acid bacteria 2.1 x 10^7 CFU/g, color (neutral-like), aroma (neutral-like), flavor (neutral-like), and overall acceptance (neutral-like). The pigeon pea sauce produced met the Indonesian National Standard and no aflatoxin B1 contamination was found in the products.

Keywords: *waru* leaf, *A. oryzae, R. oligosporus*, pigeon pea, sauce

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INTRODUCTION

Soy sauce is a liquid condiment of Chinese origin and has, over the centuries, spread to Japan and Southeast Asia (Judoamidjojo 1986; Steinkraus 1995; Xu et al. 2010; Zhong et al. 2018). Soy sauce is one of the most popular seasoning uses, especially in the Asian countries (Apriyantono et al. 1999). Indonesian soy sauce (*kecap*) is made from a fermented paste of soybeans and it has dark brown color (Nikkuni 2002).

Various ways including improvement of raw materials, processing methods, the use of pure inoculums, and controlled fermentation processes can help to increase protein content and function of soy sauce as a food seasoning. Therefore, the use of diverse raw materials and improvements in the manufacturing process are both necessary. One of the suggested raw materials for this liquid condiment is pigeon peas (*Cajanus cajan* (L.) Millsp.). Pigeon peas look so much like soybeans in their shape and size; pigeon peas also contain chemical substances that are suitable for making sauce just like soy sauce, especially the protein content (Bramel et al. 2004). Amarteifio et al. (2002) reported that pigeon peas are deficient in methionine and cysteine but high in lysine. In addition, pigeon peas are easy to grow and grow well in dry and less fertile areas that this plant may replace the function of soybeans in dry areas such as Eastern Indonesia (Dahiya 1980; Eltayeb et al. 2010; Adebole & Maliki 2011).

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Two stages of fermentation are done in the process of making soy sauce, which is the process of molds fermentation or koji fermentation and of brine fermentation. In the process of solid-state fermentation (SSF), molds play an important role because they produce various types of enzymes such as protease, amylase, and other enzymes (Yulifianti and Ginting 2018). These enzymes help to break down the basic ingredients of soy sauce such as protein, carbohydrates, and fats into amino acids and organic acids as well as flavor-forming compounds and aroma in soy sauce. Molds used in the process of molds fermentation come from various sources, one of which is *waru* leaves (Roling et al. 1994; Apriyantono et al. 1999; Nikkuni *et al.* 2002; Duniaji et al. 2014). *Aspergillus* is an important mold used for the fermentation of Asian foods; this genus is referred to as koji (Hong et al. 2015; Akpan, & Udo, 2017).

The use of *waru* (*Hibiscus tiliaceus* L.) leaves as a source of molds in the process of SSF has been popular in Indonesia resulting in a quite diverse soy sauce acceptance. However, as the process is done naturally, opportunities for the growth of various other types of unwanted molds often reduce the acceptance of produced soy sauce. Some types of molds have the ability to produce mycotoxins. *Aspergillus* sp. like *A. flavus* and *A. parasiticus* can produce very toxic mycotoxins, such as aflatoxin. Yokutsuka (1989) have found that 26 strains of Aspergilli widely used in the food industries in Japan produce mycotoxins. Mycotoxins are produced after more than 10 days of curing.

Considering the nutritional content of pigeon peas and the natural process of making pigeon pea sauce with various qualities and safety risks, it is necessary to inoculate the strains of *Rhizopus oligosporus*, *Aspergillus oryzae* and their combinations in the fermentation process of making pigeon pea sauce (Roling et al. 1994; Duniaji et al. 2014). *Aspergillus oryzae* is used in koji making due to its high productivity of hydrolytic enzymes to digest nutrients in substrates (Chancharoonpong et al. 2012; Zhong et al. 2018).

The second fermentation process is the curing process in the saline solution (commonly referred to as brine fermentation). The concentration of the saline solution used during the fermentation process can affect the acceptance of the soy sauce produced. Salt (NaCl), in addition to giving saltiness to soy sauce, is also a selective medium for the development of fermentative microbes tolerant of high salt levels. There is no uniformity of opinion related to the concentration of saline solution used for soaking. Roling et al. (1994) and Duniaji et al. (2014) suggest salt levels ranging from 30-40%, while the use of salt levels below 15% causes poor acceptance of soy sauce (Yokutsuka 1989).

Based on the aforementioned explanation, we considered the necessity to conduct research on the effect of inoculation of *R. oligosporus* and *A. oryzae* and their combination in the process of solid-state fermentation (SSF) using varied levels of saline solutions towards the characteristics of pigeon pea sauce (*C. cajan*).

MATERIALS AND METHODS

Materials

The material was local-variety pigeon peas (*Cajanus cajan* (L.) Milsp.) obtained from Tembuku Village, Tembuku District, Bangli Regency, Bali Indonesia. *R. oligosporus* and *A. oryzae* were obtained from the Inter-University Centre for Food and Nutrition, Gadjah Mada University, Yogyakarta. Palm sugar (*Arenga piñata*) and salt (NaCl) obtained from a supermarket in Denpasar City, *waru* leaves obtained from Sanur, Denpasar, and other materials for chemical analysis including NaOH, H_2SO_4, HgO, and HCl were purchased from Sigma Chemical Company (St. Louis, MO), peptone water and de Man Rogosa Sharpe (MRS) agar and PDA were purchased from Oxoid.

Research Design

The research design used was a factorial randomized block design (RBD) consisting of two factors. The first factor was the inoculum treatments, which included: (1) Control: the process of making molded soybeans or koji using *waru* (*H. tiliaceus* L.) leaves; (2) R: the process of making koji using *Rhizopus oligosporus*; (3) A: the process of making koji using *Aspergillus oryzae*; and (4) R + A: the process of making koji using *R. oligosporus* and *A. oryzae*. The second factor was soaking in a saline solution with concentrations of 15%, 20%, 25%, and 30% (w/v) and the fermented pigeon peas were grouped twice according to the processing period.

Making Pigeon Pea Sauce

The study involved the following stages. First, pigeon peas were selected to get a relatively uniform size, shape, and color, with no defects, and weighed as much as 1,000 g for each sample. Second, the peas were soaked for 15 h and drained, before being cooked using an autoclave for 60 min at a 10-15 psi. Third, the cooked beans were cooled down and then inoculated by *R. oligosporus*, *A. oryzae*, and a mix of both molds of 10^5 CFU/g each. Then, the beans went through molds fermentation process by adding *waru* leaves before incubated for 3 days at room temperature (28-30^oC) to give koji. Fourth, the koji were then dried at 40^oC for 15 min before being soaked in saline solution (brine fermentation) according to the treatment for 30 days at room temperature to produce maromi. Fifth, the pigeon peas and the resulting liquid (maromi) were separated and the filtrate was then analyzed for its protein content, pH, total acid, water content, salt content, and total lactic acid bacteria. Sixth, the filtrate was then pasteurized at a temperature of 70-80^oC for 10-15 min and seasonings were added including onion, garlic, bay leaves, and palm sugar. Seventh, the pasteurized maromi was filtered and bottled, this was the sauce product. Then, the organoleptic analysis was carried out to examine the color, flavor, aroma, and overall acceptance of the pigeon pea sauce (Sukarto 1985).

Method of Analysis

Water content was calculated using the oven method (AOAC 1995). Analysis of protein content was done employing the modified Macro-Kjeldahl method (AOAC 1995). Salinity was measured using the method from Apriyantono et al. (1989). The concentration of aflatoxin B1 in the sample was measured using the enzyme linked immunosorbent assay (ELISA) method (Chinaphuti 2003). pH was measured by referring to Indonesian National Standard (SNI 01-2891 1992). The total acid was examined using titration (AOAC 1997). The total lactic acid bacteria were measured using the method developed by Barboza et al. (2012). The sensory tests were done to the color, aroma, flavor, and overall acceptance of the sauce employing hedonic tests (Sukarto 1985) with numerical criteria: like extremely (7); like (6); like moderately (5); neutral (4); dislike moderately (3); dislike (2); and dislike extremely (1).

Statistical Analysis

All treatments in the study were performed in triplicate. The data were analyzed using SPSS statistical software (ver. 25.0 SPSS, Chicago, USA) and were subjected to two-way analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used to compare the mean. Differences at ($P<0.05$) were considered significant.

RESULTS

Water Content of Pigeon Pea Sauce

The average water content in the produced sauce with molds fermentation under room temperature (28-30ºC) is presented in **Table 1**. The results of the variance analysis showed that the interaction of salt concentration with the inoculum was not significant, the inoculum factor had no significant effect ($P>0.05$), and the salt concentration had a very significant effect ($P<0.01$) on the water content.

Protein Content of Pigeon Pea Sauce

The analysis results of protein content with salt and inoculum treatment at room temperature (28-30ºC) are presented in **Table 2**. The variance analysis showed that the interaction of treatments had no significant effect ($P>0.05$), the inoculum treatment had no significant effect, and the salt content had a highly significant effect ($P<0.01$) towards protein content, in which 20% (w/v) saline solution resulted in higher protein content of 3.50 than 15% or 25% (w/v) saline solution and the lowest protein content was obtained at 30% (w/v) salt concentration.

Table 1. Water content of pigeon pea sauce (%) made with molds fermentation under room temperature (28-30ºC)

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	Control	R	A	R+A	Average
15	84.17	83.03	85.84	83.67	84.18 a
20	84.16	81.18	80.99	80.82	81.79 ab
25	79.45	80.59	80.76	78.84	79.91 b
30	80.14	80.28	78.93	77.69	79.26 b
Average	81.98 a	81.27 a	81.63 a	80.25 a	

Means with the same letter after the average value shows no significant difference ($P>0.05$). Control: the process of making molded soybeans using *waru* (*H. tiliaceus* L.) leaves; R: *Rhizopus oligosporus*; A: *Aspergillus oryzae*; R + A: *R. oligosporus* and *A. oryzae.*

Table 2. Proteins content of pigeon pea sauce (%) made with molds fermentation under room temperature (28-30ºC)

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	Control	R	A	R+A	Average
15	1.82	2.49	2.24	1.78	2.08 b
20	3.49	3.44	3.44	3.62	3.50 a
25	2.35	2.56	2.46	2.48	2.46 b
30	1.36	1.68	1.35	1.49	1.47 c
Average	2.25 a	2.54 a	2.37 a	2.34 a	

Means with the same letter after the average value shows no significant difference ($P>0.05$). The treatment descriptions were the same as those described in **Table 1**.

Salt Content in Pigeon Pea Sauce

The analysis results of salinity with molds fermentation under room temperature (28-30ºC) are presented in **Table 3**. The variance analysis showed that the interaction of salinity and inoculum treatments was not significant ($P>0.05$), while salinity and inoculum treatments independently had a significant effect ($P<0.01$) on salt content. Each treatment was done with different salt concentration. The higher salt concentration used in brine fermentation will result in higher salt content of the sauce. The saline solution used to soak molded pigeon pea beans in brine fermentation is the liquid directly obtained from pigeon pea sauce after the waste is separated.

Aflatoxin B1 Content in the Produced Sauce

The aflatoxin analysis on the produced sauce confirmed that the treatment of salt concentration and types of inoculums no presence of aflatoxin B1. This means that molds growing during the fermentation process were not contaminated by *A. flavus* and *A. parasiticus* as aflatoxin-producing organisms. Test results of observation employing the enzyme linked immunosorbent assay (ELISA) on the produced sauce showed that the yellow color change in the microplate well as an indicator of the non-significant difference between the tested treatments compared with the standard aflatoxin B1 (0 µg/L).

pH of the Produced Sauce

The analysis results of pH with molds fermentation under room temperature (28-30ºC). The variance analysis showed that the interaction of salinity and

Table 3. Salts content of pigeon pea sauce (%) made with molds fermentation under room temperature (28-30ºC)

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	**Control**	**R**	**A**	**R+A**	**Average**
15	14.03	13.35	14.58	13.68	13.91 d
20	19.03	17.97	19.28	19.95	19.06 c
25	22.40	20.27	23.74	22.76	22.29 b
30	24.88	24.84	25.86	25.07	25.16 a
Average	20.08 ab	19.11 b	20.86 a	20.36 a	

Means with the same letter after the average value shows no significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

inoculum treatments had a significant effect (P<0.01) are presented in **Table 4**. The highest pH value of 5.42 was obtained in the combination of 25% salt content and *Aspergillus* inoculum, while the lowest pH value of 4.6 was obtained in the combination of 15% salt content and mixed inoculums of *Rhizopus* and *Aspergillus.*

Total Acid of the Produced Sauce

The variance analysis of total acid showed that the interaction of salt concentration and inoculums had no significant effect (P>0.05), while the concentration of salt and inoculums individually had a very significant effect (P<0.01) on total acid. The total acid at room temperature (28-30ºC) was around 0.36-0.62% as presented in **Table 5**. The inoculums in the control group produced the highest total acid compared to other inoculums, while the lowest total acid was for *A. oryzae* and was not significantly different from the mixed inoculum.

Total Lactic Acid Bacteria in the Produced Sauce

The variance analysis of showed that the interaction between salinity and inoculum treatments was significant (P<0.01) and that salt content and the types of inoculums had a very significant effect on the total lactic acid bacteria as presented in **Table 6**. The highest total lactic acid bacteria (LAB) of 2.6 x 10^7 CFU/g was obtained in the combination of 25% salt content and mixed inoculum, while the lowest of 1.0 x 10^7CFU/g was obtained in the combination of 30% salt and a mixed of *R. oligosporus* and *A. oryzae*. The treatments of 15% and 30% salt content in the mixed inoculum showed significant differences, but they were not significantly different from the 20% salt treatment that was equal to 2.1 x 10^7 CFU/g.

The Color of the Produced Sauce

Table 7 highlights the analysis results of the color of the produced sauce under different inoculums and salinity at room temperature (28-30ºC). The results of the variance analysis showed that the inoculum treatments were not significantly different (P>0.05), while the treatment of salt content showed significant differences (P<0.05) against the color of the produced sauce. Interaction of inoculum treatments and salt content resulted in a non-significant different effect toward the sauce color.

Table 4. pH of the produced sauce for different inoculum types and salt concentrations

Treatment	Inoculum			
NaCl salts concentration, % (w/v)	**Control**	**R**	**A**	**R+A**
15	4.85 b b	4.95 a c	4.71 c d	4.55 d c
20	5.18 a a	5.22 a b	5.19 a c	5.03 b b
25	5.15 c a	5.31 b a	5.42 a a	5.03 d b
30	5.19 c a	5.20 c b	5.34 b b	5.41 a a

Means with the same letter after the value in the same line shows a non-significant difference (P>0.05). The same letter after the value in the same column shows a non-significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

Table 5. Total acids content of produced sauce (%) made with molds fermentation under room temperature (28-30ºC)

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	**Control**	**R**	**A**	**R+A**	**Average**
15	0.62	0.59	0.53	0.52	0.57 a
20	0.56	0.52	0.40	0.43	0.48 b
25	0.53	0.46	0.36	0.44	0.45 b
30	0.57	0.47	0.43	0.40	0.47 b
Average	0.57 a	0.51 b	0.43 c	0.45 c	

Means with the same letter after the average value shows no significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

Table 6. Total lactic acid bacteria (LAB) produced sauce (x10^7 CFU g^{-1}), made with molds fermentation under room temperature (28-30ºC)

Treatment	Inoculum			
NaCl salts concentration, % (w/v)	**Control**	**R**	**A**	**R+A**
15	1.9 a b	2.0 a ab	1.3 b a	1.2 b b
20	2.5 a a	2.5 a a	1.3 b a	2.1 a a
25	1.5 b b	1.6 ab bc	1.1 b a	2.6 a a
30	1.0 a c	1.2 a c	1.0 a a	1.0 a b

Means with the same letter after the value in the same line shows a non-significant difference (P>0.05). The same letter after the value in the same column shows a non-significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

The analysis of the sauce color on the 20% salt content resulted in a score of 4.08 (neutral), which was not significantly different from the 25% and 30% salt content treatments with a score of 3.98 and 4.00, but it was significantly different from the 15% salt content treatment, with a score of 3.56 (dislike). Treatments of inoculum types showed no significant difference (the use of *waru* leaves as the source of inoculum as control and the use of the pure culture of *R. oligosporus*, *A. oryzae*, and the mixture of the two molds).

Table 7. The average value of pigeon pea sauce color

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	Control	R	A	R+A	Average
15	3.50	3.67	3.42	3.67	3.56 b
20	3.83	4.00	4.17	4.33	4.08 a
25	3.83	3.92	4.08	4.00	3.96 a
30	3.92	4.00	4.08	4.00	4.00 a
Average	3.77 a	3.90 a	3.94 a	4.00 a	

Means with the same letter after the average value shows no significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

Table 8. The average value of pigeon pea sauce aroma

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	Control	R	A	R+A	Average
15	3.58	3.50	3.33	3.58	3.50 a
20	3.75	3.92	3.92	4.00	3.90 a
25	3.83	3.50	3.67	3.42	3.60 a
30	3.75	3.50	3.42	3.33	3.50 a
Average	3.73 a	3.60 a	3.58 a	3.58 a	

Means with the same letter after the average value shows no significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

The Aroma of the Produced Sauce

The analysis results of the aroma of the produced sauce under different inoculums and salinity at room temperature (28-30ºC) were shown in **Table 8**. The results of the variance analysis showed that the interaction between inoculum treatments and the salt content did not significantly affect the aroma of the sauce. Likewise, the inoculum treatments and salt content variations individually resulted in a non-significant different effect on the sauce color (P>0.05). The average value of the sauce aroma ranged from 3.58 to 3.73 (dislike-neutral). The salt content of 15%, 20%, 25%, and 30% at room temperature (28-30ºC) showed no significant difference. The average value of sauce aroma ranged from 3.50 to 3.90 (dislike-neutral).

The Flavor of the Produced Sauce

Table 9 highlights the analysis results of the flavor of the produced sauce under different inoculums and salinity at room temperature (28-30ºC). The variance analysis showed that the inoculum treatments resulted in non-significant differences (P>0.05), while the salt content treatments showed significant differences (P<0.05) of the sauce flavor.

The comparison between the treatment of using *waru* leaves (control), with the treatment of *R. oligosporus* and *A. oryzae* and the combination of the two, no significant difference was found in the flavor of the sauce (**Table 9**). The average value of the sauce flavor ranged from 3.50 to 3.63 (dislike–neutral). The treatment of 20% salt content with a score of 3.96 was not significantly different from the treatment of 25% salt content; yet, it was significantly different from the treatment of 15% and 30% salt content, with a score of 3.48 and 3.27. The average value of the sauce flavor ranged from 3.27 to 3.96 (dislike-neutral).

Table 9. The average value of pigeon pea sauce flavor

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	Control	R	A	R+A	Average
15	3.08	3.33	3.25	3.42	3.27 b
20	3.92	4.00	3.83	4.08	3.96 a
25	3.58	3.58	3.42	3.58	3.54 ab
30	3.42	3.50	3.58	3.42	3.48 b
Average	3.50 a	3.60 a	3.52 a	3.63 a	

Means with the same letter after the average value shows no significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

Table 10. The overall average value of pigeon pea sauce acceptance

Treatment	Inoculum				
NaCl salts concentration, % (w/v)	Control	R	A	R+A	Average
15	3.58	3.75	3.42	3.75	3.63 b
20	4.00	4.33	4.33	4.50	4.29 a
25	3.75	3.92	4.00	3.92	3.90 ab
30	3.58	3.58	3.67	3.42	3.56 b
Average	3.73 a	3.90 a	3.85 a	3.90 a	

Means with the same letter after the average value shows no significant difference (P>0.05). The treatment descriptions were the same as those described in **Table 1**.

Overall Acceptance of the Sauce Organoleptic

The analysis results of the organoleptic of the produced sauce under different inoculums and salinity at room temperature (28-30ºC) were shown in **Table 10**. The variance analysis showed that the inoculum treatments resulted in non-significant differences (P>0.05), while the salt content treatments showed significant differences (P<0.05) of the overall acceptance. The interaction of inoculum treatments and salt content resulted in a non-significant difference of the overall acceptance. **Table 10** shows that between the treatment of using *waru* leaves (control), with the treatment of *R. oligosporus* and *A. oryzae* and the combination of the two, no significant difference was found in the overall acceptance of the sauce. The average value of the overall acceptance ranged from 3.73 to 3.90 (dislike-neutral). The treatment of 20% salt content was not significantly different from the treatment of 25% salt content; yet, it was significantly different from the treatment of 15% and 30% salt content. The average value of the overall acceptance ranged from 3.50 to 3.90 (dislike-neutral).

DISCUSSION

The water content of the produced sauce ranged from 79.26% to 84.18% at various concentrations of salt. The higher the concentration of the saline solution used, the lower the water content would be. In concentrated solutions, the amount of solid content is higher than the amount of water. The microbial activity is influenced by water content in the material, but the activity does not take or produce much water that the inoculum factor does not affect changes in water content in the material. The produced sauce, stable at room temperature, which does not require refrigeration during storage due to its low water activity (a_w) and high salt content (Luh 1995).

In brine fermentation, salt (NaCl) acts as a preservative and selective medium for microbes that are important during fermentation. Salt-tolerant or halophilic microbes can develop well at the appropriate salt concentration, while high concentrations of salt inhibit such process (Yokutsuka 1994). Duniaji et al. (2014) suggests that 20% NaCl concentration is a good preservative for enzyme extracts from *Aspergillus* sp., and, conversely, the addition of 25% NaCl can inhibit the proteolytic activity. Inoculum treatments, both individuals and mixed, did not significantly affect the protein produced, showing that the same ability to form protease enzymes. Fermentation in molded soybeans can eliminate the unpleasant odor of soy caused by the activity of lipoxygenases. The fungus that plays a role in the fermentation process is *R. oligosporus* (Weng and Chen 2011; Hong et al. 2012). Yokutsuka (1989) states that koji making or molded soybeans forms proteases, amylases, and lipases. Rejeki et al. (1994) writes that molds that play a role in SSF are *A. oryzae*, *A. niger*, and *Rhyzopus* sp. According to Zhong et al. (2018) and Wang et al. (2010), *Aspergillus oryzae* was used in koji making due to its high productivity of secreting various hydrolytic enzymes to break down large molecules during 3-day solid-state fermentation. Zhao et al. (2018) reported that *Aspergillus oryzae* is an excellent mold for soy sauce fermentation because of its complicated enzyme system, especially protease. Several other regulatory mechanisms may also be responsible for increasing overall protease production (Zhong et al. 2018). The production of enzymes by *A. oryzae* S. is related to the physical properties of soybean koji during fermentation (Chancharoonpong et al. 2012). Weng and Chen (2011), Surono (2016) and Pilco et al. (2019) suggest that *Rhizopus oligosporus* is known as tempeh mold, which has beneficial properties because, in addition to being proteolytic and lipolytic, it is also capable of producing antibiotic substances against Gram-negative bacteria that are pathogenic. Salt is a medium of selection for microbes that develop in brine fermentation and is very influential on the activity of proteases formed in molds fermentation. Salt can cause proteins, including enzymes, to undergo denaturation (salting in or salting-out) that it will affect the activity of proteases to break down proteins into dissolved. Based on the Indonesian National Standard, the quality requirement for soy sauce is 2.0 to 6.0% protein content and 1.35% to 4.04% protein content for soy sauce with SSF under room temperature (28-30ºC). Thus, it can be concluded that the produced sauce meets the Indonesian National Standard.

The type of inoculum influences the salt content; the highest salt content in this study was obtained in the *Aspergillus oryzae* inoculum, but it was not significantly different from the mixed and control inoculums. The lowest salt content was obtained in the *Rhizopus oligosporus* inoculum, but it was not significantly different from the control inoculum. The treatment with high salt concentration will lead to the high salt content in the produced sauce. All treatments of salt concentration resulted in significant differences; this was because the saline solution became the sauce solution during brine fermentation after the waste was separated, and the salt only acted as a selective medium.

The type of molds growing on *waru* leaves was the species from *Rhizopus* sp. (Duniaji et al. 2014), so this type of mold has a more dominant role in a natural molds fermentation process. The result illustrates that the yellow color shown on the microplate well is proportional to the yellow color on the standard 0 µg/L aflatoxin. The darker yellow color indicates a lower concentration of aflatoxin B1, while the brighter yellow color indicates a higher concentration of aflatoxin (Chinaphuti et al. 2003). Aflatoxins are toxic fungal metabolites that pollute various agricultural products (Kolosova et al. 2006). In this experiment the testing of aflatoxin B1 using ELISA kits gave negative results for all treatments, therefore the resulting product was safe and in accordance with standards. It is suspected that the molds in the *waru* leaves as well as the two types of molds used in this experiment were able to suppress the growth of the fungus *Aspergillus flavus* which commonly grows on legume beans.

Compared to controls, *Rhizopus* or *Aspergillus* treatment at variations in salt concentration resulted in a higher pH, while the inoculum mixed treatment resulted in a lower pH except at 30% salt concentration. The microbial control that developed in molds fermentation was more varied than the pure inoculum; this allowed acid-forming microbes to develop and produce acid. Kasmidjo (1990) asserts that at the beginning of the salt fermentation process, *Lactobacillus delbrueckii* bacteria actively form lactic acid, whereas a higher salt concentration produces a higher pH that inhibits the growth of microbes so acid-forming bacteria are inhibited. Chancharoonpong et al. (2012) reported that during koji fermentation, the soybean koji pH increase was caused by enzymes production. As stated by Axelsson (2004), the salt content of 20% can become an inhibitor, while at 25% concentration causes inhibition in proteolytic enzymes. Yokutsuka (1994) affirms that halophilic bacteria can thrive at salt concentrations of 5-20%, while at 20-30% their development is inhibited.

In the control inoculum (*usar*), the types of microbes that existed diverse including acid-forming microbes, so during the fermentation process, both molds fermentation and brine fermentation, acid was formed more quickly. The 15% salt treatment produced the highest total acid content, while the 20% to 30% salt treatment resulted in a non-significant difference in the total acid; this means that salt had become a selective medium for microbes. At salt concentrations of more than 15%, the growth of acid-forming microbes is inhibited. Salt-tolerant or halophilic microbes can develop well at the appropriate salt content, yet high salt content inhibits their development (Yokutsuka 1994).

Saline solution is a selective medium for the growth of lactic acid bacteria. The microbial growth competition is quite high at low salt content, yet selection happens at high salt content, in which microbes that grow mainly are lactic acid bacteria (Adebole and Maliki 2011; Barboza et al. 2012). Yokutsuka (1994) reported that halophilic bacteria can develop well and optimally at 5-20% salt content, yet the growth of these bacteria is inhibited at 20-30% salt content.

The sauce color, in addition to being influenced by the color of its natural ingredients, is also closely related to the process of making it. In general, the color of soy sauce is blackish brown; this color can occur during the fermentation process and during the last processing, where the heating process (pasteurization) causes browning reaction on soy sauce. Wu et al. (2010) reported that the brine color at 25°C changed into a darker color after two days of fermentation. Differences in the mold activity also affect the activity of the enzymes produced, so the components of the fermentation process are related to the release of color-forming compounds, both the natural color of the raw material and the color formed during the soy sauce processing. One important property is the color of soy sauce, with recent evidence showing consumer preferences for lighter-colored soy sauce products for particular dishes. The natural soybean fermentation microbial community, can be engineered to reduce the ‘brownish’ reaction during soybean production. There are two approaches to produce ‘de-browning’: consumption of engineered xylose, important precursors in browning reactions, and engineering of degradation of melanoidin, which is the main brown pigment in soy sauce. Both of these strategies can work synergistically by using mixed culture to produce better de-browning (Det-udom et al. 2019).

According to Harada et al. (2013), the aroma compounds generation and changes in sensory characteristics through fermentation of koji soy sauce. Sensory analysis showed a marked increase in ‘musty’ and ‘soy sauce’ smells, while the beany attribute decreased significantly during koji fermentation. Aldehydes and alcohols are the main volatile classes present in koji samples. Feng *et al*. (2013) reported that the microbes influence the presence of aroma constituents and chemically influenced by lactic acid, acetic acid, and ethanol. It was further stated that the lactic acid and a bacterium associated with the main aromatic compound, 2,5-dimethyl-4-hydroxy-3 (2H)-furanone. However, most of the aroma constituents change with the presence of lactic acid bacteria and the resulting acid, which means that the effect of lactic acid and acetic acid on the aroma profile is significant. Complex microbial interactions on two stage fermentation play an important role in the development of flavor. Zhong et al. (2018) state that under the conditions of conidiation and fermentation, it is enhanced hydrolytic enzyme production and flavor precursor formation. Efforts to enhance and accelerate the formation of flavor in the presence of certain salt concentrations and inoculation with a mixture of starter cultures, namely: *A. oryzae* and *R. oligosporus* can provide better organoleptic quality. Flavor development in pigeon pea sauce was significantly related to the diversity of microbes.

CONCLUSION

The treatment of mixed inoculum and 20% salt content in molds fermentation resulted in the best pigeon pea sauce characteristics. The characteristics of the pigeon pea sauce were as follows: 80.33% water content, 4.04% protein content, 19.41% salt content, pH 6.2, 0.22% total acid, 2.1 x 10^7 CFU/g lactic acid bacteria, color (neutral-like), aroma (neutral-like), flavor (neutral-like), and overall reception (neutral-like). The produced pigeon pea sauce met the Indonesian National Standard and no aflatoxin B1 contamination was found in the product.

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