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PERUBAHAN PROTEIN DAN HIDROGEN SIANIDA PADA PEMBUATAN TEMPE KACANG KORO PEDANG (*Canavalia ensiformis* (L) DC)

Protein and hydrogen cyanide changes during jack bean (Canavalia ensiformis (L) DC) tempe processing

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Abstrak
Kacang koro pedang (*Canavalia ensiformis* (L.) DC) merupakan salah satu jenis kacang-kacangan yang dapat dimanfaatkan sebagai bahan baku pembuatan tempe namun juga mengandung hidrogen sianida. Terdapat beberapa tahapan pengolahan pada pembuatan tempe kacang koro pedang yang dapat menurunkan kadar hidrogen sianida namun meningkatkan kualitas protein kacang koro pedang. Penelitian ini dilakukan dengan tujuan untuk mengetahui perubahan kadar hidrogen sianida, protein terlarut dan protein total selama proses pengolahan tempe kacang koro pedang. Perlakuan diberikan pada setiap tahapan proses pengolahan tempe kacang koro pedang dengan total enam tahapan yaitu bahan baku, perendaman, perebusan pertama, perebusan kedua, inokulasi, dan fermentasi. Variasi konsentrasi inokulum dan lama fermentasi juga dikaji dalam penelitian ini. Hasil penelitian menggambarkan kadar hidrogen sianida dan total protein menurun sejalan dengan proses pengolahan tempe kacang koro pedang. Kadar protein terlarut meningkat pada tahap perendaman kemudian menurun pada tahap perebusan dan meningkat dari tahap inokulasi hingga menjadi tempe kacang koro pedang. Seiring meningkatnya konsentrasi inokulum dan waktu fermentasi menurunkan kadar hidrogen sianida namun meningkatkan kadar air dan protein terlarut. Penggunaan inokulum 1,5% (b/b) menghasilkan total protein tertinggi dibandingkan yang diperoleh dari konsentrasi inokulum 1,0% dan 2,0% dengan waktu fermentasi 24 dan 48 jam.

Kata Kunci: tempe, kacang koro pedang, inokulum, waktu fermentasi, hydrogen cyanide

Abstract
Jack bean (*Canavalia ensiformis* (L.) DC) is a bean that can be used to make tempe because its protein althought contains hydrogen cyanide. There are several processing steps during the tempe making of jack bean, that can ameliorate hydrogen cyanide but improve protein quality of jack bean. This research was conducted with the aim to determine changes in levels of hydrogen cyanide, soluble protein and total protein during the processing of jack bean tempe. The treatment was applied on each stage of the processing of jack bean tempe with a total of six stages, namely raw material, 24 h soaking, first boiling, second boiling, inoculation, and 24 h fermentation. Hydrogen cyanide and total protein gradually lowered in line with the jack bean tempe processing, respectively. Soluble protein levels increased at the soaking stage then decreased at the boiling stage and increased from the inoculation stage to become jack bean tempe. The higher inoculum concentrations and the longer fermentation time caused hydrogen cyanide decreased. In reverse, moisture and soluble protein content increased. Using 1.5 % (w/w) inoculum produced the highest total protein content compared to those obtained from 1.0% and 2.0% inoculum concentration both in 24 and 48 h fermentation time.

Keywords: tempe, jack bean, inoculum, fermentation time, hydrogen cyanide

PENDAHULUAN

Tempe is a traditional Indonesian food that is widely consumed and contains high levels of nutrients. It is made from soybeans most of which are imported because of soybean shortage in Indonesia to meet domestic needs. To solve it, efforts were being made to use other types of local beans grown in Indonesia. One of

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non-soy legumes that is potential as a basic raw material for making tempeh is jack bean.

Jack beans (*Canavalia ensiformis* (L) DC) is a local leguminous plant that is rich in protein and carbohydrates (Akpapunam & Sefa-Dedeh, 1997; Sutedja et al., 2020), but is still not widely used by Indonesians. The jack beans also contains anti-nutrients, namely concanavalin A, trypsin inhibitors, toxic non-protein amino acids (canavanine and canaline), saponins, cyanogenic glycosides and polyphenols (Akande, 2016). The decomposition of cyanogenic glycosides releases hydrogen cyanide (HCN), which is dangerous to human health when it enters the body. The HCN level contained in raw jack bean seeds is 264.38 ppm (mg/kg beans) (Murdiati et al., 2017).

The HCN content in a food product needs to be limited because it be highly toxic. The acute toxicity effects of cyanide on humans vary depending on its concentration. If it is consumed, the acute lethal oral dose of cyanide in humans is reported to be between 0.5 and 3.5 mg/kg body weight (Schrenk et al., 2019). Hydrocyanic acid level, including hydrocyanic acid bound in cyanogenic glycosides with a cassava approach of a maximum of 50 mg/kg of material (*Regulation - 2022/1364 - EN - EUR-Lex*, 2022)

One of the food processes that can reduce HCN is fermentation (Bamidele et al., 2015; Essers et al., 1995; Hawashi, et al., 2019; Kumoro et al., 2020; Lambri et al., 2013; Obi et al., 2019; Omolara, 2014; Zvauya & Muzondo, 1995). The fermentation process can also convert complex nutritional compounds into simpler ones, e.g., protein that is hydrolyzed into soluble proteins thus they become more easily digested by human body. The fermentation process in making tempe is closely related to the characteristics of inoculum and its fermentation lengths. Inoculum is defined as a collection of microorganisms; where for tempe are generally fungi from genus Rhizopus sp. which is the key microorganisms to making tempe. Rhizopus sp. carries out metabolic activities during fermentation process by hydrolyzing complex molecules into simpler compounds, including cyanogenic compounds. The hydrolysis of cyanogenic compounds will release HCN which will evaporate due to heat energy from the fungi metabolic process.

Inoculum concentration and fermentation length determine the kinetics of the fermentation process where optimization of these two factors to carry out metabolic activities give a high value of importance in food fermentation technology. Research on making jack bean tempe (Diniyah et al., 2014) stated that variations in the percentage of inoculum (1.0,1.5, and 2.0 %) had a significant effect on the protein content. A short time course of fermentation causes the fungi to be incapable of optimizing the ongoing metabolic activity. Fermentation lengths of 24-36 h is generally accepted as the exponential phase of optimum growth of fungi, while the fermentation lengths of 36-48 h is the deceleration phase, so that the fermentation time course more than 48 h brings about the fungi experiencing a death phase, which causes a rotten smell or off-odor, furthermore, the texture of the tempeh being too soft (Wahyudi, 2018).

The decrease in HCN also occurs due to several types of processes other than fermentation, such as soaking (Hawashi, Sitania, et al., 2019; Kresnadipayana & Waty, 2019) and boiling (Ojiambo et al., 2017; Quinn et al., 2022). By knowing the potential of each influencing factor, making tempe from jack bean also can be hypothesized that there is opportunity to reduce HCN because of these key stages in making tempe. In this research, there are 6 stages of the tempe processing process where the changes will be examined, i.e., raw material, soaking, first boiling, second boiling, inoculation, and fermentation.

The fungi that grow during fermentation will of course also use the protein for metabolism in the jack bean seeds as the raw materials. Therefore, the aim of this research is to evaluate each stage of the tempe making process and their effects on the hydrogen cyanide, total protein and soluble protein content of jack bean tempe. An in-depth examination at fermentation stage is what the roles of the combination of inoculum concentration and fermentation length.

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MATERIAL AND METHODS

Materials and Reagents

The jack beans obtained from local farmers in Temanggung, Central Java, Indonesia. Inoculum was commercial *Rhizopus sp*, produced by Indonesian Institute of Sciences (LIPI), Bandung. Reagents for analysis were $AgNO_3$, HNO_3, KSCN, $NH_4Fe(SO_4)_2.12H_2O$, NaCl, K_2CrO_4, Kjeldahl tablets, H_2SO_4, HCl, methyl red indicator, methyl blue indicator,

ethanol, Zn powder and H_3PO_4 were purchased from Merck, Germany. Coomassie Brilliant Blue G-250 and Bovine Serum Albumin fraction V purchased from AppliChem, Germany. Na_2CO_3 purchased from Himedia, India. NaOH purchased from Tjiwikimia, Indonesia.

Jack bean tempe making processing

Jack beans (**seeds**) were sorted to separate incomplete and damaged seeds. Washed seeds were soaked into boiling water in 1:4 weight ratios of the seeds for 24 hours (**24 h soaking**) and drained. The seeds were then washed, boiled in water (1:4 weight ratio) at 95°C for 30 minutes (**1st boiling**), peeled, reduced in size, and boiled again at 95°C for 15 minutes (**2nd boiling**). Then inoculated (**inoculation**), packaged in perforated plastic and fermented at room temperature (**tempe**). Inoculum concentration of 1.5% and fermentation length of 24 h were used in the evaluation of fermentation effects on the jack bean tempe processing. Samples from the inoculation stage were analyzed one hour after the inoculum was mixed into the jack bean seeds. The evaluation of the influence of the process stages was carried out as the first stage of research, while in the second stage the influence of the combination of inoculum concentration and fermentation time was evaluated. A combination of concentration variations (1%; 1.5%; and 2% w/w) and fermentation lengths (24 h and 48 h) were carried out to determine the influence of these two factors to the jack bean tempe.

Moisture and total protein contents

The determination of moisture and total protein content was carried out referring to AOAC (Horwitz, 2005). Nitrogen levels were corrected by reducing the nitrogen value of the cyanide compounds present in the sample. Total protein content is expressed as a percent of dry weight.

Soluble protein content

The determination of soluble protein content of jack beans tempe was carried out using the Bradford method (Kruger, 1994). Samples (2 g) were extracted with distilled water (4 mL) and separated using a centrifugate (4°C; 6,000 rpm; 15 min). The protein extract (100 μL) was added with Bradford reagent (5 mL), then vortexed and incubated for 5 minutes. The appearance of bluish color of the sample solution was measured using a spectrophotometer (Shimadzu UV-1900, Jepang) for their absorbance values at 595 nm. The amount of soluble protein was calculated against a calibration curve of bovine serum albumin as the standard solution and presented as a percentage of dry weight.

Hydrogen cyanide content

Hydrogen cyanide analysis was referred to the research conducted by (Putro et al., 2015). Finely ground sample (10 g) was added with distilled water (100 ml) and kept for 2 h. Added another distilled water (100 ml) was added and distilled it. The distillate was collected by reacting using $AgNO_3$ 0.02N (20 ml) and HNO_3 (1 ml). The distillation was stopped after the distillate reached 100 ml and filtered. Excess $AgNO_3$ in the distillate was titrated with KCNS 0.02 N using the ferric ammonium alum indicator. Standardization of the $AgNO_3$ solution is carried out with 0.02N NaCl solution. Standardization of the KCNS solution was carried out with 0.02N $AgNO_3$ solution. The cyanide content was expressed as percent of dry weight.

Data analysis

All assays of the experiments were carried out in triplicates. The results of all assays were expressed as mean values and standard deviation (SD). To determine the effect of each processing steps and the combination of inoculum concentration and fermentation time, data for all parameters were analyzed using ANOVA ($p<0.05$) and continued with multiple comparison tests using Duncan's Multiple Range Test (DMRT) at $p<0.05$. Data of soluble protein content, total protein, and hydrogen cyanide from each stage of the process were applied to Past 4.03 software (Hammer et al., 2001) to perform principal component analysis (PCA) which mapped the existing relationship among different processing step of jack bean tempe. Pretreatment of the data was done by calculating a ratio to its average, prior to PCA analysis

RESULT AND DISCUSSION

Jack beans contained relatively higher protein than other types of beans such as mung beans dan red kidney beans. This opens up a great opportunity to use these beans as raw material for making tempe. However, the high hydrogen cyanide content was a barrier to its use, in particular, safety aspect; therefore, it warranted evaluation of the tempe processing stages and determining the appropriate inoculum concentration and fermentation length. Chemical changes of the jack bean characteristics along

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with tempe making process were observed included the parameters of moisture, hydrogen cyanide, total protein, and soluble protein content.

Moisture content

Changes in moisture contents during the processing of jack bean tempe were shown in Fig. 1. The soaking stage made the cell walls of the beans absorbing water (increased in moisture content) which then imbibed the cells and macromolecules resulting in larger sizes and softer textures. During the boiling process, the water content of the beans continued to increase. It was related to the changes of the bean matrices after boiling observed under a scanning electron microscope (SEM) and of the bean density (Sutedja et al., 2022). After soaking, the beans underwent 1st boiling, by which the water content increased by 8.53% then it increased by 24.93% due to 2nd boiling (Fig. 1). These explained that water hydration into the beans was greater during 2nd boiling even though the boiling time was only 15 minutes because the beans had been peeled and cut it into 6 parts so the total surface areas of the beans became higher.

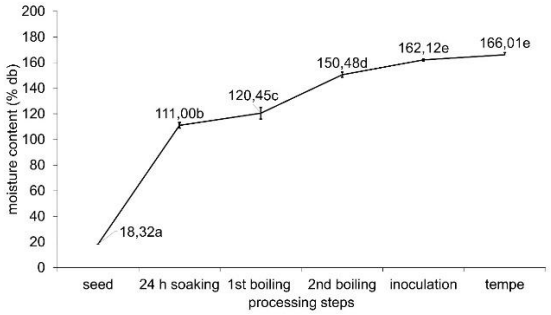

Figure 1. Changes in moisture content s (% db) during the process of jack bean tempe processing applied fermentation length of 24 hours (n=3). Values were expressed as mean ± SD. Values followed by different letters within were significantly different ($p<0.05$).

During the inoculation process, tempe inoculum was added in the form of dried Rhizopus sp. powder and it was mixed into the boiled, peeled, and smaller bean particles. The short contact time of the inoculated inoculum and the test time caused the fungi on the mixture to grow and experienced initial respiration activity. Respiratory activity broke down complex molecules into simpler compounds with the help of limited oxygen (facultative aerobic atmosphere in the packs) and the substrate most needed for respiration was carbohydrates in the form of starch. Starch was broken down into simple sugars, i.e., glucose which then being oxidized to Krebs cycle became pyruvic acid. Further metabolism converted it into carbon dioxide, water and a certain amount of energy as Adenosine Triphosphate (ATP). After 24 h fermentation, it produced tempe with higher moisture content, which was about 2.40% from the inoculation stage, because it had just entered the exponential growth phase.

The moisture contents of jack bean tempe at different inoculum concentrations did not differ significantly at 24 h fermentation, meanwhile, in 48 h fermentation, different inoculum concentrations had a significant effect (Fig. 2). As fungi entered the exponential growth phase, it started to use the substrate in the fermentation media (bean kernels). The moisture content of jack bean tempe significantly increased at 48 h of fermentation along with the increase in inoculum concentration compared to that at 24 h of fermentation with the same concentration. These data explained that considerable metabolism occurred during fermentation in the second 24 hours after inoculation.

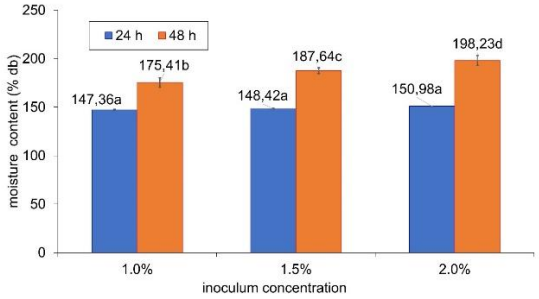

Figure 2. Moisture content (% db) of jack bean tempe in combination of inoculum concentration (1.0, 1.5 and 2.0%) and fermentation time (24 and 48 h) (n= 3). Values were expressed as mean ± SD. Values followed by different letters within were significantly different ($p<0.05$).

Hydrogen cyanide (HCN)

Fig. 3. described HCN contents undergo further decreasing and it continuously takes place until the jack bean converts into tempe with total reduction by 54.21%. The initial beans had very high levels of HCN and when the soaking process was carried out, the levels increased by around 47,27% due to spontaneous fermentation by microbes, mostly lactic acid bacteria, during the soaking process. Those bacteria that grow spontaneously utilized sugar compounds resulting from the hydrolysis of

existing cyanogenic glycosides so that hydrogen cyanide was released. The soaking process started when hot water contacted the bean surfaces. High temperatures made the hydration process into the kernel bean faster, causing expansion of the bean matrices and making it easier for HCN to dissolve into bulk water.

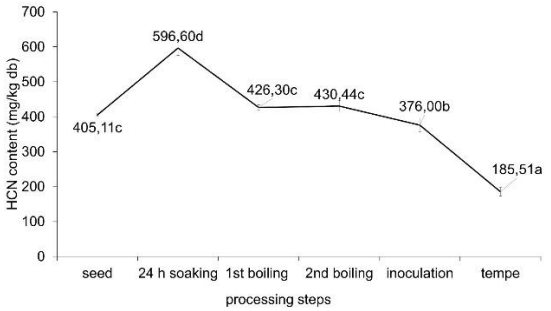

Figure 3. Changes in Hydrogen cyanide (HCN) content after the jack bean tempe fermented for 24 hours (n=3). Values were expressed as mean ± SD. Values followed by different letters within were significantly different ($p<0.05$).

Boiling process was carried out twice to eliminate the HCN of the jack bean. The higher the temperature used in boiling stage, the higher the decrease in HCN content in the beans. The HCN content decreased by 31.57% 1st boiling compared to those in the soaking process and it decreased by 12.53% after 2nd boiling. The smaller reduction levels HCN in 2nd boiling compared to those in 1st boiling, indicating level off removal power, due to much more free cyanides had been released and evaporated during 1st boiling. The heat penetration process expanded the structural matrix in the beans, causing the HCN diffused out more easily into the boiling water so that reduced HCN levels in the beans. The presence of glucosidase enzyme in the bean also catalyzed the conversion of cyanogenic glycosides to HCN during 1st boiling. On the other hand, in 2nd boiling, the seed coats of the jack beans had been removed and the kernel sizes were reduced in size to approximately pieces with dimensions of 0.5x0.5 cm which made heat penetration from the boiled water into the kernel greater because the surface area of the seeds was higher and no more seed coat barrier. This caused the temperature inside the seeds to increase more quickly and made the enzymes inactivate more quickly.

Fig 4. described that after 24 hours of fermentation, as the inoculum concentration increased, the HCN content of jack bean tempe decreased. The further decrease in HCN levels in jack bean tempe was caused by the increasing concentration of the inoculum, so that more and more fungi would utilize the nitrogen element instead of protein from HCN (Wahono et al., 2016).

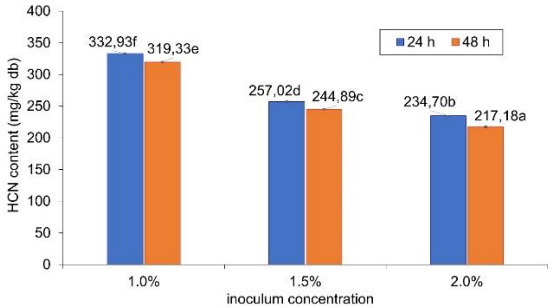

Figure 4. HCN contents (% db) of jack bean tempe due to combination of inoculum concentrations (1.0, 1.5 and 2.0%) and fermentation lengths (24 and 48 h) (n=3). Values were expressed as mean ± SD. Values followed by different letters within were significantly different ($p<0.05$).

Fermentation length of 48 h with increasing inoculum concentration reduced the HCN content of jack bean tempe. The decreasing HCN content were due to the fungi having more time to utilize non-protein N elements, so the HCN levels will decrease further. The HCN content of jack bean tempe for fermentation of 48 h with an inoculum concentration of 1.0 % was higher than for fermentation of 24 h with an inoculum concentration of 1.5 %. This explained that a 1.0 % concentration with a fermentation length of 48 h reached the exponential phase required a longer time course to degrade HCN compared to those with a higher inoculum concentration and a shorter fermentation length. Longer fermentation period, reduced the toxicity component more (Essers et al., 1995).

The decrease in HCN occurred because the fungi used the non-protein N element from HCN. The use of non-protein N elements by the fungi also had an effect on protein levels. Fungi hydrolyzed complex molecules into simpler ones, such as cyanogenic glucosides which were broken down into glucose and aglycone-cyanogen, so that glucose were hydrolyzed into water, among others. The complex macromolecule proteins were also hydrolyzed into simpler molecules which were easily soluble in water, so that increasing the inoculum concentration and fermentation length will increase the soluble protein content of jack bean tempe.

Total protein and soluble content

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The total protein relatively remained constant from the raw materials to the boiling process (Fig. 5a) while the soluble protein was increased during soaking then reduced until tempe produced (Fig 5b). The increase in soluble protein content in the soaking process increased by 218.52 % compared to the raw beans. The growth of lactic acid bacteria during soaking involved respiration which used oxygen and protein in the beans played as building block source for reproduction and increased the number of cells so that the total protein in the beans decreased. Proteolytic enzymes degraded the proteins into simpler molecules which was more soluble and detected as the increment of soluble protein. The proven fermentation during the soaking process were indicated by the changes of soaking water becoming cloudy and foamy (surfactants derived from proteins).

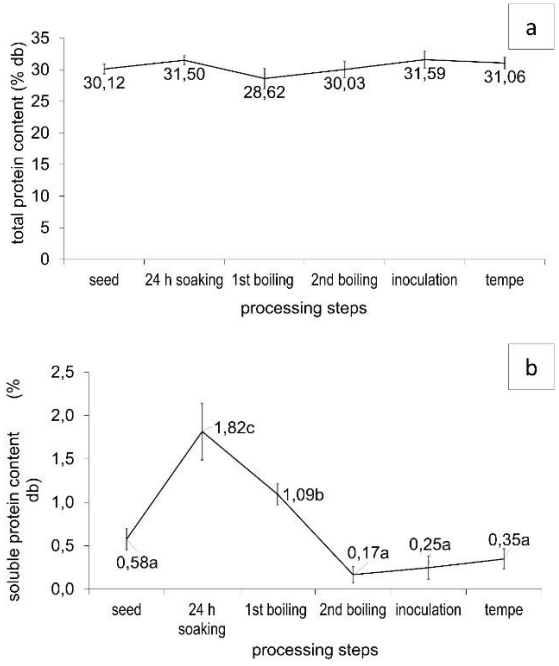

Figure 5. Changes in total protein content (a) and soluble protein content (b) during the process of jack bean tempe processing applied fermentation length of 24 hours (n=3). Values were expressed as mean ± SD. Values followed by different letters within were significantly different ($p<0.05$).

When first and second boiling processes were carried out, the soluble protein content in the beans decreased by 118.94% from the soaking process because much of the protein was dissolved in the cooking water and during boiling the protein denaturation process occurred (Fig. 5b). Proteins with its hydrophilic properties will easily dissolve into the cooking water and some of proteins will experience denaturation due to heat during boiling. The occurrence of protein denaturation caused some of the weak chemical bonds in the protein structure to open and the structure of the protein complex changed slightly but the primary structure remained intact. Opening this structure caused the hydrophilic side of the protein to be exposed so that it dissolved more easily in water.

During fermentation process, protein in the beans will be hydrolyzed by fungi into other nitrogenous compounds (N). This was used by fungi as a building block source for growth and reproductions to increase the number of cells which was characterized by the more mycelia and forming a compact bean tempe. In this study, the protein content of jack bean tempe after 24 h of fermentation decreased by 6.86% from the inoculation stage whereas the soluble protein increased by 7.11% because of protease activity of the fungi inoculants. This protease degraded complex protein molecules into simpler compounds, thereby turning them into soluble proteins. However, the increase in total protein levels was not significantly different from the inoculation stage.

There was a real influence of variations in inoculum concentration, variations in fermentation time, and the interaction of the two on the total protein and the soluble protein content of jack bean tempe (Fig 6a and 6b). A fermentation time of 24 h with varying concentrations gave significantly different results with an increase in dissolved protein levels with each increase in inoculum concentration. Soluble protein levels after 24 h of fermentation with concentrations of 1%, 1.5%, and 2% decreased. This shows the presence of soluble proteins that were used by fungi for respiration, reproduction and metabolic activities. The 24-h fermentation time was the initial phase of the exponential stage or adaptation of the fungi to the substrate, so that the fungi use the simple compounds of jack bean seeds in the form of simple sugars, starch and also soluble protein first for metabolic activity. Soluble proteins were simple proteins that were easier for fungi to use for metabolic activities, especially during this exponential phase.

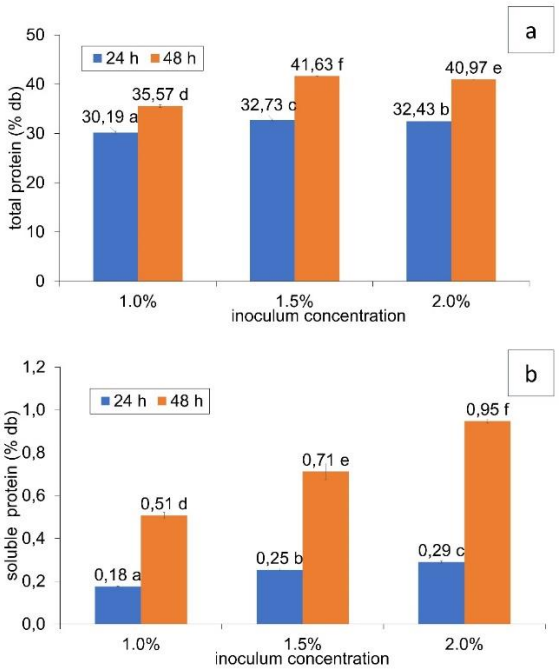

Figure 6. Total protein and soluble protein content (% db) of jack bean tempe in combination of inoculum concentration (1.0, 1.5 and 2.0%) and fermentation lengths (24 and 48 hours) (n=3). Values were expressed as mean ± SD. Values followed by different letters within were significantly different ($p<0.05$).

Fermentation time of 48 h with increasing inoculum concentration showed an increase in dissolved protein levels. The increase in soluble protein levels occurred because the fungi was in the exponential phase, so it can carry out optimal activities to degrade the components that make up the jack bean seed, including hydrolysis of protein into simpler components, namely proteases, peptones, peptides and amino acids. An increase in the inoculum concentration at 48 h of fermentation caused an increase in dissolved protein levels, because the number of fungi increased to hydrolyze complex proteins into soluble proteins. The conversion into amino acids affects the distinctive flavor of the tempeh produced.

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was used to determine the relationship between process stages and changes in protein and HCN content during the manufacture of jack bean tempe. Based on Fig. 7, it was known that treatments soaking 24 h and 1st boiling, play a major role in changing the composition of soluble protein and total protein content of jack bean seeds. The soaking step made soluble protein increased and 1st boiling reduced its soluble and total protein content. The HCN content parameters were closely related to the process stages of 2nd boiling, inoculation and fermentation. The position of HCN which was in the opposite quadrant to those processing steps explained the negative correlation between them and explain that 2nd boiling, inoculation and fermentation played a major role in reducing HCN levels.

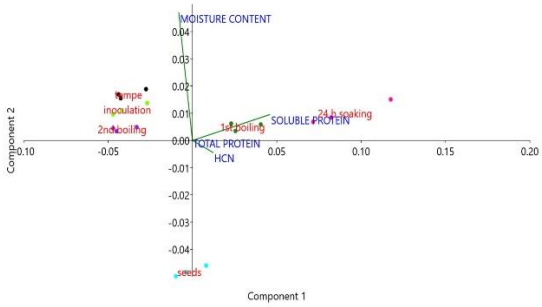

Figure 7. Bi-plot of principal component analysis (PCA) of jack bean tempe processing steps, describing 79.48% of data variation for Component 1 and 16.04% for Component 2.

CONCLUSION

Processing steps of jack bean tempe greeted changes of moisture, HCN, total protein, and soluble protein content. The moisture content was increased as much as 806.13% compared to the raw material. The HCN and total protein content were decreased, respectively. The soluble protein content increased after soaking stage compared to the raw material, which then decreased due to first and second boiling; then increased reaching 40.96% compared to that in inoculation stage when finished becoming jack bean tempe. Varying inoculum concentrations increased the moisture and soluble protein content. Using 1.5 % of inoculum, both run for 24 and 48 h fermentation, made jack bean tempe reached the highest total protein content compare those from jack bean tempe using 1.0% and 2.0 % inoculum. Thus, the recommended stages of the jack bean tempe making process were soaking for 24 hours, first boiling, second boiling, inoculating with 1.5% inoculum, and fermentation for 24 h.

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