POTENCY OF AMINO ACIDS AS SAVORY FRACTION FROM VEGETABLE BROTH OF MUNG BEANS (Phaseolus radiatus L.) THROUGH BRINE FERMENTATION BY Rhizopus-C1

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ABSTRACT

Amino acids produced through brine fermentation of mung beans (Phaseolus radiatus sp) by inoculum of Rhizopus-C1 at room temperature for 0, 2, 4, 6, 8, 10, and 12 weeks, respectively had a potential use as savory fraction for seasoning agent. The objective of this experiment was to find out characteristic of produced amino acids and composition of fermentation products relating with proteolytic and amylolitic activities of Rhizopus-C1. The result of experiment showed that the length of fermentation time would increase intensity of savory taste and cloudy color, and increase total protein, soluble protein, and N-amino concentrations, decrease water, while fat concentration was constant. Fermentation of 10 weeks was optimal time to get crude broth with concentrations of total protein of 9.5622%, soluble protein of 8.5 mg/g, N-amino of 5.6 mg/g, fat of 0.2802%, water of 40.7189%, Volatile Reduction Substances (VRS) of 90 µg/g, and reduction sugar of 672.5 mg/mL. Kinds of dominant non-essential amino acids produced were glutamic acid (1.014%), and aspartic acid (0.507%), while essential amino acids were lysine (0.474%), and isoleucine (0.644%). The other of amino acids were resulted with concentration of 0.211 – 0.345%, such as leucine, arginine, serine, glycine, histidine, alanine, proline, tyrosine, valine, methionine, cystine, threonine, and phenylalanine. Visually, crude vegetable broth produced through brine fermentation of mung beans by Rhizopus sp-C1 was semi solid, brownish color, rather fatty, salty, and enough strong savory taste.

Keywords: Amino acids, brine fermentation, mung beans broth, Rhizopus-C1, savory fraction

INTRODUCTION

Mung bean fermentation (Phaseolus radiatus L.) is a development process in fermentation food industry as vegetable broth for seasoning agent. The choice of mung bean is based on almost indifferent concentration of protein (24%) to another local legume, such as red bean (29.1%). Use of Rhizopus sp. inoculum in brine fermentation is an alternative effort to get savory flavor besides Aspergillus sp. inoculum which is mainly used in brine fermentation-based in preparation of foods, such as ketchup, taucu [1]. Rhizopus sp. usually is used as inoculum in production of tempeh. Enzymes produced by inoculum for fermentation process (protease, amylase, lipase) will convert and degrade legumes to simpler compounds affecting on characteristic of savory flavor. Proteolytic activity of inoculum and quality of substrate protein has the important role in recovering amino acids as precursor savory flavor [2].

Amino acids are precursor of savory flavor, non-volatile compounds, are reached through synthesize and natural fermentation methods. Amino acids resulted through brine fermentation of legumes had been well-known as precursor of savory flavor, such as ketchup, taucu (Indonesia), Miso and Katsuobushi (Japan), Bagaoong and Tao-si (Philippine), Meju (Korea), and Prahoc (Cambodia) [3]. These amino acids were generally produced by using mixed inoculums of Aspergillus oryzae, Aspergillus sojae and less Rhizopus sp. as microorganism in resulting enzyme which will convert components in legumes to volatile and non-volatile compounds in forming savory (umami) taste. Utilization of Rhizopus-C1 in brine fermentation is caused by a potential exploration as inoculum in preparation of tempeh supported by non-essential amino acids, particularly glutamic acid (7.353 mg/100 gram dry weight) of total non-essential amino acids (44.221 mg/100 gram dry weight of tempeh [4].

The objective of this experiment was to find out the potential use of amino acid and the product composition through brine fermentation at room temperature for 0, 2, 4, 6, 8, 10, and 12 weeks relating with proteolytic and amylolitic activities of Rhizopus-C1 using mung beans substrates supporting its important role as savory flavor.

EXPERIMENTAL SECTION

Material

Materials used in this experiment were mung beans purchased locally, starter Rhizopus-C1 (Research Centre for Chemistry, Indonesian Institute of Sciences), reagent for proximate and dissolved protein [6], and amino acids analyses [7].

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Instruments

Equipments utilized in this work were autoclave, incubator, brine fermentation system in laboratory scale, Spectrophotometer UV-1201 and HPLC (Waters 2487, USA).

Procedure

Experimental design and Analyses

This experiment was employed by using inoculum Rhizopus-C; starter at room temperature for 0, 2, 4, 6, 8, 10, and 12 weeks of fermentation in laboratory scale (100 - 150 g). Analyses were carried out on proteolytic (Walter) and amylolytic (Somogy-Nelson) [5] activities of inoculum of Rhizopus-C. Analyses of composition were employed on raw material of mung beans and product of vegetable broth covering water content (Gravimetric), total protein (Kjeldahl), dissolved protein (Lowry), fat (Soxhlet), reducing sugar (Somogyi-Nelson) [6], and N-amino (Cu method) [7]. At the best time of fermentation was reached by analysing and identification on amino acids (HPLC instrument) [8].

Identification of Amino acids

Analysis was carried out by weighing 0.1 g crude broth of fermented mung beans, diluting to 5 - 10 mL of HCl 6 N and heating at 100 °C for 24 h. After filtration, 50 mL of sample was added with 50 mL Methanol and 50 mL of derivatization solvent (Triethylamine), allowed for 20 min, added with acetonitrile of 0.5 - 1 mL, and injected to HPLC (Waters 2487, USA) using Picosas column of amino acid with wavelength of 254 nm at flow rate of 1 mL/min and injection volume of 20 µL. Concentration of amino acids in sample calculated from chromatogram, was expressed in µmol of amino acids (µmol AA), and was determined as percentage of amino acids (weight/dry weight of protein). Chromatogram, preoper from standard concentration and sample concentration of amino acids was presented in Figure 9, 10 and 11. Calculation is expressed in formula, as follow:

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\% AA = \frac{\text{Peak area of sample}}{\text{Peak area of standard}} \times \frac{\text{Standard Concentration}}{\text{Sample weight}/100} \times \frac{\text{MW} \times \text{Dilution} \times 100}{100}
\]

Process Steps

Preparation of inoculum of mung beans broth.
Starter of Rhizopus-C was added to rice substrates which had been rinsed overnight, autoclaved at 120 °C for 15 min, cooled and incubated at 35 °C for 72 h with concentration of 0.2% (w/v). This mixture was then dried at 50 °C for 24 h in cabinet dryer, powdered by grinder, screened through 80 mesh sieve and ready to be used as inoculum in the process of brine fermentation.

Preparation of vegetable broth of mung beans.
A number of mung beans was washed, rinsed overnight, dehulled, autoclaved at 121 °C for 20 min, and cooled.

Sterilized mung bean was then added to salt in ratio 51% and 23%. Broth inoculum of Rhizopus-C, of 26% was aseptically added to the mixture and fermented at room temperature up to 12 weeks in closed jar in which each week was investigated and analyzed. Agitating and transferring jar was aseptically carried out every week.

RESULT AND DISCUSSION

Characteristic of broth inoculum from starter of Rhizopus-C

Rhizopus sp. which had been utilized as inoculum in preparation of tempeh has savory taste due to their amino acids and peptides concentrations. In this brine fermentation, starter of Rhizopus-C, which is Rhizopus oligosporus is isolated from lari is a fermenting agent added to make tempeh [9]. It is source of enzymes (protease, amylase and lipase) generating components formation of savory flavor. Starter inoculum of Rhizopus-C possesses a optimal growth for 56 h of incubation sign by growing dense micella. Figure 1 (a) and 1 (b) represented the growth of Rhizopus-C during incubation in rice substrates at optimal time of 56 h (a), and dried inoculum after drying at 50 °C for 24 h (b). The growth of Rhizopus-C during 56 h of incubation results dense does not utilize initial sporulation. Enough high activities of proteolytic (1.14 Uni/g) and amylolytic (6 Unit/mg) enable to be reached a product with composition and specific volatile components. These high activity of enzymes are caused by incubation time, temperature, humidity, purity of starter, and inoculum. Inoculum composition with concentrations of carbohydrates (71.5%) and total protein (17.19%) are source of nutrition and enzymes of inoculum. Concentrations of dissolved protein (6.95 mg/mL) and N-amino (2.61mg/g) in inoculum give a sufficient high contribution on formulation of vegetable broth. In this brine fermentation, inoculum concentration affects directly on the end composition of product because inoculum (26%) is directly used as formulator in final product, while in formulation of ketchup is added palm suiker and spices indicated as condiment [10].

Composition of mung beans

Mung beans is sources of carbohydrate, fat and protein of this main product. High concentration of protein (25.3%, dry weight) enables to be yielded amino acids and high dissolved peptides due to protease activity of Rhizopus-C, whereas carbohydrate (62.12%) which is source of starch will be hydrolyzed by α-amylase enzyme into monosaccharide through Maillard reaction which will naturally produce specific taste. The presence of fat (0.47%) will be
Figure 1. The growth of *Rhizopus-C₁* during incubation at rice substrates for optimal time of 56 h (a) and dried inoculum as result of a drying at 50 °C for 24 h (b).

![Figure 1](image1.png)

Composition:
- Water: 9.69%
- Ash: 2.42%
- Fat: 0.47%
- Carbohydrate: 62.12%
- Protein: 25.30% (dry weight)

Figure 2. Mung beans commodity and their compositions as raw material in brine fermentation to prepare *vegetable broth* as savory flavor.

![Figure 2](image2.png)

Figure 3. Relationship between fermentation time and water content and total protein of *vegetable broth* from mung beans using inoculum *Rhizopus-C₁* at room temperature in laboratory scale.

![Figure 3](image3.png)

During brine fermentation, demand of water to grow and metabolite of inoculum is enough large so it will drop water content of product. Inoculum of *Rhizopus-C₁* will be active at 28 – 35 °C [11]. This range of temperature will be reached because substrates in closed jar can occur an increase of inoculum activity and a demand of water to grow and produce enzymes. In this decrease of water content, flavor intensity will become more and more high due to product thickness.

Figure 4 (a), (b), and (c) indicated crude broth produced via brine fermentation of mung beans using inoculum *Rhizopus-C₁* at room temperature for 12 weeks (a), substrates as semi solid mass, brownish and rather wet (b), and paste broth (c).

![Figure 4](image4.png)

The effect of brine fermentation on broth composition of mung beans

Brine fermentation generated water content which tends to decrease, but total protein tends to rise, displayed in Figure 3.
weeks, substrates as semi solid mass, brownish and rather wet, and paste broth, respectively. On increase of total protein concentration, significant raise seemed up to 4 weeks of fermentation, and then tend to 12 weeks of fermentation. This increase is depend on initial concentration of total protein before fermentation, so during fermentation, *Rhizopus-C₁* realize possibility the growth and increase of enzyme production. Increase the inoculum and enzyme give a contribution in raising total protein because inoculum is also a potential source of protein. At 4 weeks of fermentation, total protein becomes constant indicating no more difference of total protein in substrates because it occurred equilibrium reaction of fermentation between enzymes production and deamination process into amino acids, peptides, and flavor components.

Fermentation process increase also dissolved protein and N-amino relating with the length of time of fermentation, showed in Figure 5. Sharp increase of dissolved protein seemed at 6 weeks of fermentation (10.69 mg/mL) and then drop to 12 weeks of fermentation (8.6 mg/mL). The same trend was shown in amino acids concentration as N-amino, in which a linear increase seemed to 6 weeks of fermentation (5.6 mg/mL), fluctuated, and became more and more high to the final fermentation (7 mg/mL). Raise of dissolved protein takes place to 6 weeks of fermentation followed by a decline at 12 weeks of fermentation.

This condition was possibility caused by the optimal proteolytic activity of *Rhizopus-C₁* at 6 weeks of fermentation so protein and protease enzymes stock in substrates become more and more low decreasing dissolved protein. Increase of N-amino is still continuing to 12 weeks of fermentation, which is an indication of their formations of taste and specific aroma by contributing amino acids, such as glutamic acid.

*Rhizopus-C₁* produced acid, alkaline, and neutral proteases with metabolic activity at the optimal temperature of 30 – 37 °C, and the different range of substrates pH. Protease enzyme will hydrolyze chain of protein peptides into amino acids and simpler peptides so it might change orientation of all protein molecules, namely side chain of hydrophobic, non-polar is arranged to inner surface, and side chain of polar hydrophobic is outer section to increase their solubility in water as polar solvent [12].

The presence of salt in this fermentation can increase production and protease activity by increasing free amino acids concentration [13]. Inoculum of *Rhizopus-C₁* is a single isolate of *Rhizopus oligosporus* [9] having high activity of proteolytic in preparation of tempeh and results tempeh’s aroma which is not strong with longer life time [14]. Its application on brine fermentation enables to be produced specific flavor due to lower interaction among species so flavor resulted is not uniform. In other words, flavor resulted is affected by reactions of other microbes, as well.

The length time of fermentation will increase reducing sugar concentration, while volatile components formed tend to be constant, shown in Figure 6.

Sharp increase of reducing sugar takes place at 6 weeks of fermentation (809 mg/mL), and then drop to the final fermentation (583.75 mg/mL). This matter is relating with amilolytic activity of inoculum in hydrolyzing glycoside binding from a carbohydrate chain at inoculum of rice substrates (26%) and mung beans (51%) into monosaccharide having reducing sugar group, such as glucose. In fermentation range of 4 to 6 weeks, amilase activity is possibility very reactive, and then decrease because all their activities used have drop reducing sugar.
Figure 7. Relationship between fermentation time and fat of vegetable broth of mung beans using inoculum Rhizopus-C₁ at room temperature in laboratory scale.

Besides, it is enabled to convert glucose to energy through glycolysis reaction for growing inoculum or it takes place a conversion of glucose into alcohol and organic acids by enzyme activity of inoculum to result flavor. A number of monosaccharide react also with amino acids through Maillard reaction in order to form melanoidin pigment and a number of intermediate components (furan, pyrazine, etc.) contributing on taste and specific aroma [15], displayed in Figure 4 (b). During fermentation, enzymes (protease, amilase, and lipase) hydrolyze carbohydrate, protein, and fat contained in substrates to form volatile components accumulating to yield specific flavor. Volatile Reduction Substances (VRS) is a volatile compound which can be oxidized, such as alcohol, aldehyde, ester, hydrocarbon and other organic compounds. The prolong time of fermentation can cause an increase or decrease of VRS concentration. The highest concentration of VRS takes place at 2 weeks of fermentation, namely 105 μg/g.

On total fat concentration, longer time of fermentation tends to produce broth with fluctuation concentration of fat, and decrease to 12 weeks of fermentation, displayed in Figure 7. Fat content of this fermentation product is relating with fat of mung beans (0.47%) and broth inoculum (1.8%) in which for 0 - 12 weeks of fermentation, fat content changes to 0.2572 – 0.0824%. Change in fat concentration is affected not only by lipolytic activity, but also by water content in substrates, in which more and more high water content will increase lipase activity. It had been indicated that lipase enzyme has 5 folds of activity at water content of 15% than water content of 9.8% [16] so water content in substrates ranging of 40.7189 – 44.2961% will cause sufficient high activity of lipolytic to convert fat content of product to fatty acids and glycerol. Rhizopus sp. has high activity of lipolytic indicated in production of tempeh in which hydrolysis of lipase in degrading fat to unsaturated fatty acids can reach 20 – 22% [17] and has a potency in forming flavor.

The effect of brine fermentation on recovery of amino acids

Analyses of amino acids are carried out at 10 weeks of fermentation according to sensory aspect. From kinds of dominant amino acids, essential amino acid (histidine, arginine, isoleucine, leucine, lysine, methionine, phenilalanine, threonine and valine), particularly isoleucine (0.644%), leucine (0.437%) and lysine (0.474%) are kinds of amino acids with the highest concentration. Figure 8 represented a chromatogram profile of amino acids in crude broth produced through brine fermentation for 10 weeks with a chromatogram sequential consisting of aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cystine, isoleucine, leucine, phenilalanine and lysine as shown in peak no. 7, 9, 11, 14, 16, 18, 20, 23, 25, 27, 29, 33, 34, 36, 37 and 39. Essential amino acids (histidine, arginine, isoleucine, leucine, lysine, methionine, phenilalanine, threonine and valine) are obtained in range of 0.252 – 0.644% (protein, dry weight), while non-essential amino acids (glutamic acid, glycine, proline, tyrosine, cystine, aspartic acid, alanine, and serine) range 0.211 – 1.014% (protein, dry weight) in concentration dominated by glutamic acid (1.014%), aspartic acid (0.907%) and cystein (0.4%) (see Table 1). These concentrations of acids are affected by initial material and result of degrading of
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