Fermentation of soybean (*Glycine max (l.) merr*) using mix inocula of *Rhizopus sp*. and *Sacharomycescereviceae* for alternative source of folic acid

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Fermentation of soybean (*Glycine max (l.) merr*) using mix inocula of *Rhizopus sp*. and *Sacharomycescereviceae* for alternative source of folic acid

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Abstract. Folic acid (folate) is a water-soluble B vitamin needed by human body for enhancing metabolism and developing body cells. Soybean contains relatively high folate, but it needs cooking process –that may cause a loss of folate– to be edible. Therefore, folate content in soybean can be re-enhanced by fermentation to produce tempe using *Rhizopus sp*. as inoculum. Development of inoculum by mixing *Rhizopus sp*. with *Saccharomyces cerevisiae* yeast was performed to obtain higher folate content. This study was conducted to understand the effects of inoculum ratio in enhancing folic acid content. *Rhizopus sp*. and *Saccharomyces cerevisiae* at ratio of 1:0, 1:1, 1:2, and 1:3 were tested for 0–72 h fermentation time with interval of 8 h at 30 ℃, at a concentration of inoculum of 0.2 wt-% of soybeans. Results showed that interactions of treatment processes with inoculum ratio and fermentation time affected mycelial growths, tempe appearances, overall compositions, and folate contents. The optimum process conditions based on dissolved protein and folate content (1.67 mg/mL and 381 µg/mL, respectively) was at a ratio of 1:3 for 72 h.

1. Introduction

High-quality of human resources (HR) determines the future. Thus, healthy mothers are needed to give a birth of healthy babies with good intra-uterine and extra-uterine growth. Good quality of human resources is influenced by a very important organ, the brain, approximately 78% of human brain growth occurs during intra-uterine, and the rest is up to 2 years. Various congenital abnormalities in infants, including incidence of neural tube defect (NTD), premature delivery, low birth weight and impaired brain growth during intrauterine can be caused by the deficiency of some essential nutrients [1]. One substance needed to prevent the abnormalities is folic acid. According to the Institute of Medicine [2] [3], the intake of folic acid for brain development in pregnancy is in the range from 400 µg per day, which will affect the brain growth by 80 percent compared to other nutrients. The contribution of folic acid is not only useful for fetal brain development from embryo tangible, but it also becomes an essential key to the growth of healthy brain function during pregnancy [4].

Folic acid (or folate) is a compound with a molecular weight (Mw) of 441. Folate consists of three groups, namely pteridine, a ring containing a nitrogen atom, the ring psoriasis aminobenzoic acid (PABA) and glutamic acid [5]. Folic acid is a kind of vitamin B group, which is one of important elements in the synthesis of DNA (Deoxyribo nucleic acid) to provide assistance in the metabolism and

the development of body cells. The human body cannot synthesise folate structure, therefore it requires intake from food [6]. Folate is widely available in various types of plants and animal tissues, especially as polyglutamate in the form of reduced methyl or formal [7].

There are numerous foods with rich folate content, such as yeast, liver, kidney, leafy green vegetables, cauliflower, broccoli, beans and other food sources that have sufficient amounts, such as foods made from milk, meat, fish, and less contain in the fruits [8]. However, since folate is thermolabile and soluble in water, it is normally damaged due to cooking process [5]. Heating can destroy about 50–90% of folate in the food. Raw soybean is one of the most abundant sources of folate. However, it has less to utilize directly due to its high oil content, low digestibility and rotten smell (green beany) [9]. Therefore, further processing is required. Soaking and cooking are well-known cooking steps in soybean processing. Extended period cooking process causes a significant loss of folic acid [10]. Maintaining folate in food, efficient alternative methods are required, and one of them is fermentation. Fermentation with *Rhizopus*has been reported to increase the folate content of soybean [11].

The fermentation process of soybeans into tempeh have several advantages; such as higher digestibility and essential amino acids, lower anti-nutritive substances, such as trypsin inhibitors and phytic acid. According to Codex Alimentarius, tempeh also has the organoleptic quality standard, such as: 1). It has compact texture and it does not easily crumble by cutting with a knife, 2) It has white color from the growth of *Rhizopus sp*., 3) It has flavors, such as nutty, meaty and mushroom-like smell, 4) It has a fresh aroma without odor [12].

During fermentation process in the production of soybean tempeh, decomposition and simplification of existing components in soybeans into smaller and simpler occur. Therefore, tempeh will be easily digested and absorbed by the body. Those simplifications are biocatalyzed by enzymes produced by microorganisms. Microorganisms play a key role during the fermentation are molds. According Purwijatiningsih et al [12], the type of mold found on commercial starter culture (laruRaprima) is *Rhizopus sp*., including *Rhizopusoligosporus*. The use of *Rhizopus sp*. as an inoculum consisting of mixtures of several *Rhizopus sp*. is a mandatory in tempeh fermentation. *Rhizopusoligosporus* plays a major role in the making of tempeh. Yeast is able to grow during tempeh fermentation and its growth can promote mold growth on tempeh and transform the appearance and flavor of tempeh. Kustyawati [13] reported that Saccharomyces boulardii yeast was inoculated with *R. oligo*sporus in soybean for tempeh fermentation. The addition of the yeasts to the fermentation was reported to contribute the increase of folic acid content during incubation time. Without yeast addition, the prepared tempeh contained 72.76 μg folic acid/100 g of dry tempe. While, by the addition of yeast, folic acid content was 89.28 μg/100 g of dry tempeh. The yeast was reported to produce lipolytic and proteolytic extracellular enzymes abundantly.

Exploration of potential inoculum is fundamentally an important concern to obtain tempeh with more specific functions. This work investigated the development of tempeh inoculum by combination of mold inoculum *Rhizopussp* with yeast *Sacharomycescerevisiae* (baker's yeast). Mold inoculum mixture consists of *R. oligosporus, R. stolonifer, R. oryzeae* and *R. arrhizis.* Preparation of inoculum by the addition of yeast *S. cerevisiae* was conducted to obtain a higher folic acid in produced tempeh from soybean.

2. Materials and Methods

2.1. Materials

Materials used in this study were soybean from local market and Inoculum of *Rhizopusoligosporus-sp*isolate (Research Center for Chemistry – LIPI, Raprima, AFI), *S.cereviceae* (Commercial Brand Fermifan), All chemicals and solvents used for process and analysis were of analytical grade and purchased from local distributors.

2.2. Instrumentation

Process apparatus used in this experiment were microbiology equipment such as laminar air flow, water bath, incubator, fermenter, homogenizer, grinder, and cabinet dryer. The main analysis instruments were spectrophotometer UV-Vis and LC-MS.

2.3. Experimental Design

Experimental work was conducted to ferment soy beans at temperature 30 ºC for 0–72 hours with sampling each 8 hours. with condition variation as follow, (1) *Rhizopus sp*. Inoculum combining with yeast *S.cereviceae* at a ratio of 1:0, 1:1, 1:2 and 1:3, respectively. (2) fermentation time from 0 to 72 hours with intervals of 8 hours for sampling. Analysis was done for total solids (gravimetry), dissolved protein (Lowry), N-amino (Cu method), and folic acid (spectrophotometry). Furthermore, folic acid oligomer was identified by LC-MS (Mariner Biospectometry) with LC (Hitachi L6200). All samples were conducted in triplicate and all data were processed in descriptive analysis based on average result of analysis.

2.4. Methods

2.4.1. Process Fermentation steps of soybean

Few amount soybeans were cleaned, then soaked in water for 24 h at pH of 5.5. This step caused an increase of beans volume. The beans were then separated from its skin, and steamed for 30 min, cooled and inoculated with *Rhizopus sp.* inoculum mixture (*R. oligosporus, R. stolonifer, R. oryzeae, and R. arrhizis*) with concentration of 0.2% (w/w beans) by combining with yeast *S.cereviceae* at a ratio of 1:0, 1:1, 1:2 and 1:3, respectively. Incubation was performed for each mixture at 30ºC for 0–72 h with an interval of 8 hours and visual observations of tempeh fermentation conditions with combination of *Rhizopus sp.* and yeast *S. cereviceae.*

2.4.2. Analysis of folic acid

Folic acid analysis was performed using spectrophotometric method based on the reaction of diazotisingp-aminobenzoylglutamate acid produced after the reduction reaction of folic acid and 3-aminophenol to form a complex of yellow-orange. About 1 mL of standard folic acid or sample was added into 1 ml of 4 M HCl , 1 mL of 1% (w/v) sodium nitrite, 1 mL of 1% (w/v) sulfamic acid, and 1 mL of 1% (w/v) 3-aminophenol. After mixing, it formed a yellow-orange complex solution. Furthermore, the absorbance was measured using UV–VIS spectrophotometer at a wavelength of 460 nm [15].

2.4.2. Folic acid identification by LC-MS

After fermentation process, fermented soybeans extract were then filtered by microfiltration. Identification of folic acid and glutamate acid compounds in soybean fermentation at the optimum condition. The obtained permeate or purified tempeh extracts and folic acid standard. Oligomer analysis was performed by LC-MS using Mariner Biospectrometry integrated with Q-tof mass spectrometer (MS) through ESI (electrospray ionisation) system where the scan mode shown in the range of 100-1200 sqm at 140□C. C18 column Supelco (RP 18, 250 x 2 mm with a particle size of 5 µ) was used for the LC (Hitachi L 6200). Solvent was a mixture of water containing 0.3% acetic acid (A) and methanol containing 0.3% acetic acid (B) at a ratio of 80% methanol and 20% water with a flow rate of 1 mL/min. The injection volume was 20 □l [16].

2.4.5. Statistical analysis

The statistical analysis was done using Minitab Statistical Software, Release 16 for Windows., for orthogonal regression equation and the one-way analysis of variance procedure.The ANOVA was used to determine whether significant ($P<0.05$) variation occurred among means in each experiment. The Tukey's honest significant difference was used to determine which means differed significantly. Experiments were conducted in triplicate. Each value was the mean of all three independent trials (n=3).

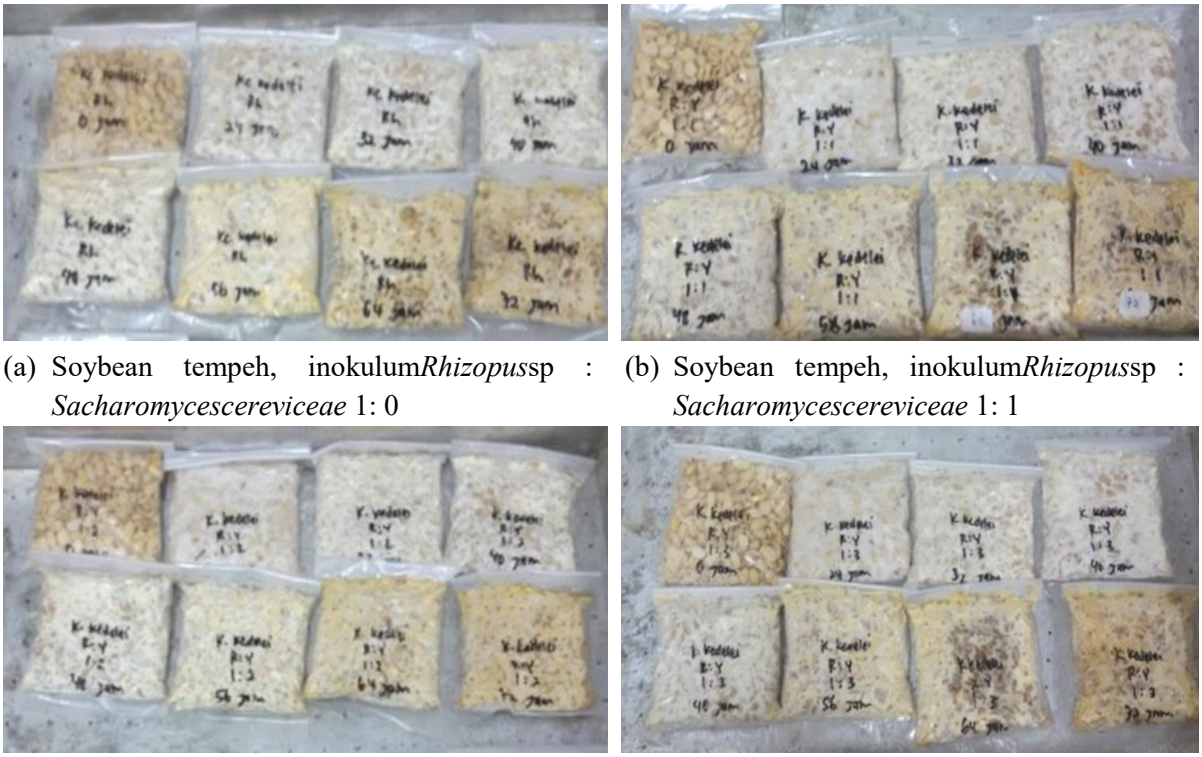
3. Results

Chemical characteristic of soybean and visual observations of tempeh fermentation conditions with combination of *Rhizopus sp*. and yeast *S. cereviceae.*The results were shown below (Table 1

Table 1. Chemical characterization of soy beans (*Glycine soja* L.)

Composition	Kind of material	
	Soybean soak in water	Steamed soybean
Water content (%)	62.16	39.31
Dissolved Protein (mg/mL)	3.42	1.47
N-amino (mg/mL)	3.92	2.24
Folic acid (μg/mL)	1606.37	286.74

Visual appearances of soybeans without and with yeast in various ratios were shown in Figure 1



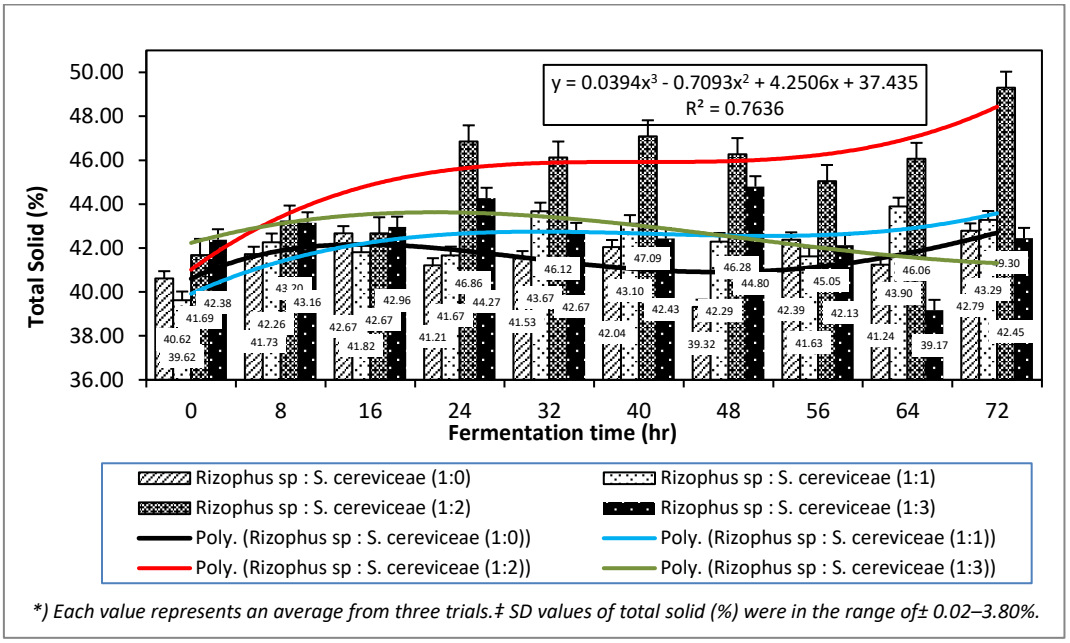
(a) Soybean tempeh, inokulum*Rhizopus*sp : *Sacharomycescereviceae* 1: 0

(b) Soybean tempeh, inokulum*Rhizopus*sp : *Sacharomycescereviceae* 1: 1

(c) Soybean tempeh, inokulum*Rhizopussp* : *Sacharomycescereviceae* 1:2

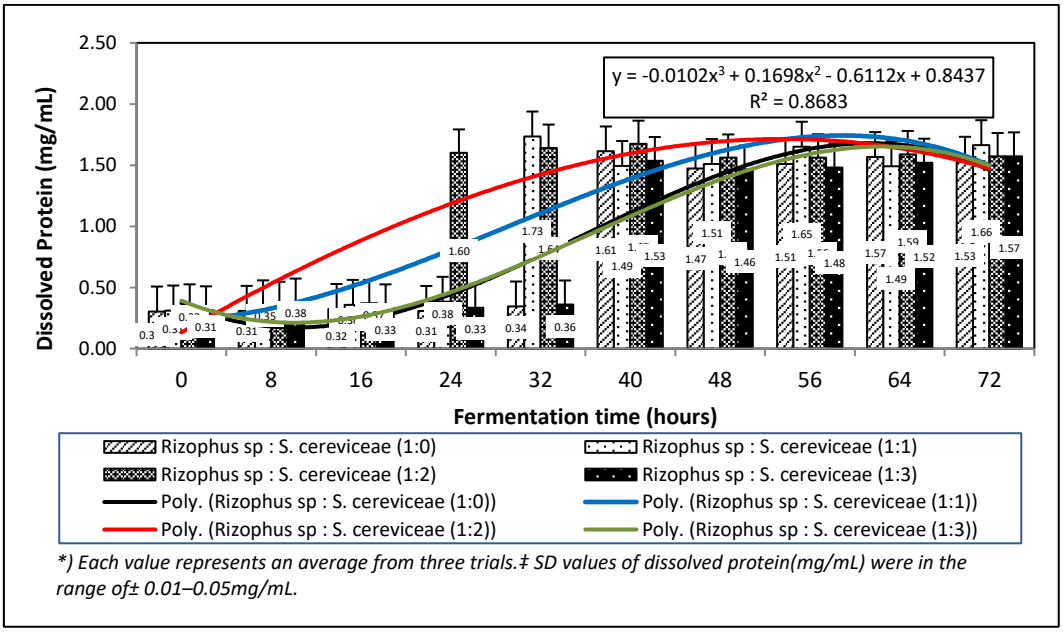
(d) Soybean tempeh, inokulum*Rhizopussp*: *Sacharomycescereviceae* 1:3

Figure 1. Visual appearances of soybean tempeh withdifferent ratio of *Rhizopus sp*. and *Sacharomycescereviceae*, at 30ºC, for 0–72h with an interval observation time of 8 h.



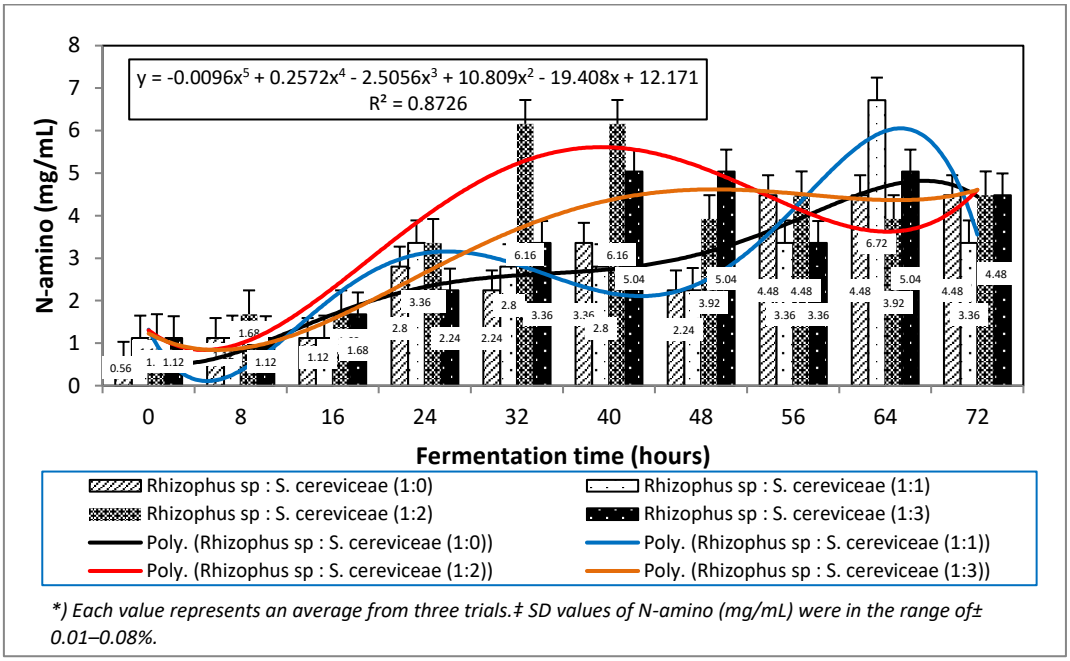
*) Each value represents an average from three trials.‡ SD values of total solid (%) were in the range of± 0.02–3.80%.

Figure 2. Effects of fermentation time and inoculum ratio with isolates of *Rhizopus sp*.and*Sacharomycescerevicae* to total solids of fermented soybeans



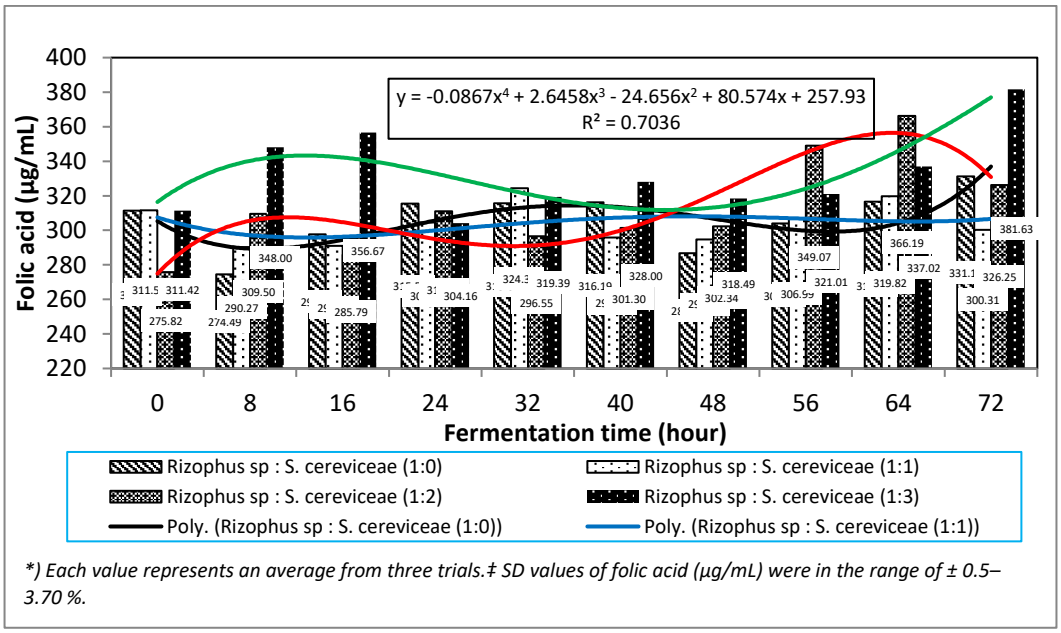
*) Each value represents an average from three trials.‡ SD values of dissolved protein(mg/mL) were in the range of± 0.01–0.05mg/mL.

Figure 3. Effects of fermentation time and inoculum ratio with isolates of *Rhizopus sp*. and *S. cerevicae* to dissolved protein of fermented soybeans



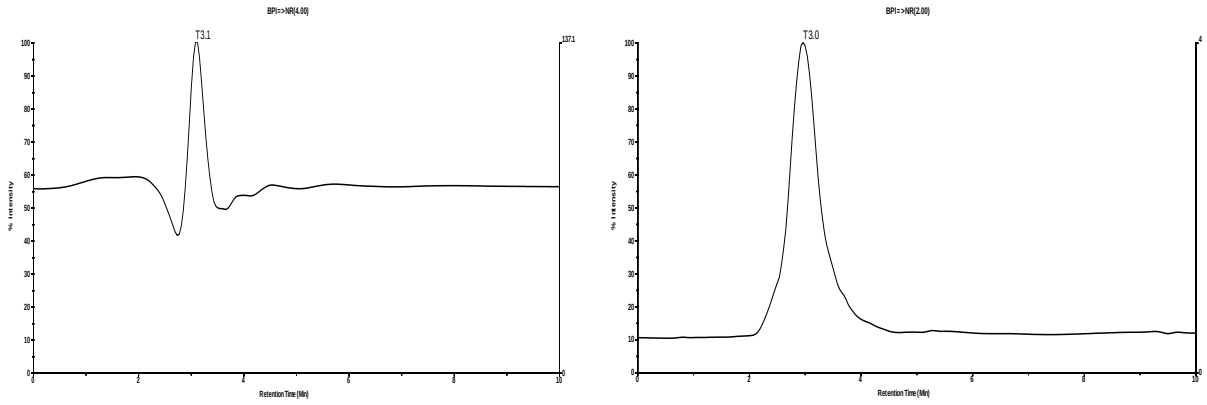
*) Each value represents an average from three trials.‡ SD values of N-amino (mg/mL) were in the range of± 0.01–0.08%.

Figure 4. Effect of fermentation time and the ratio of inoculum and isolates of *Rhizopussp.*and*S. cerevicae*to N-amino of fermented soybeans.



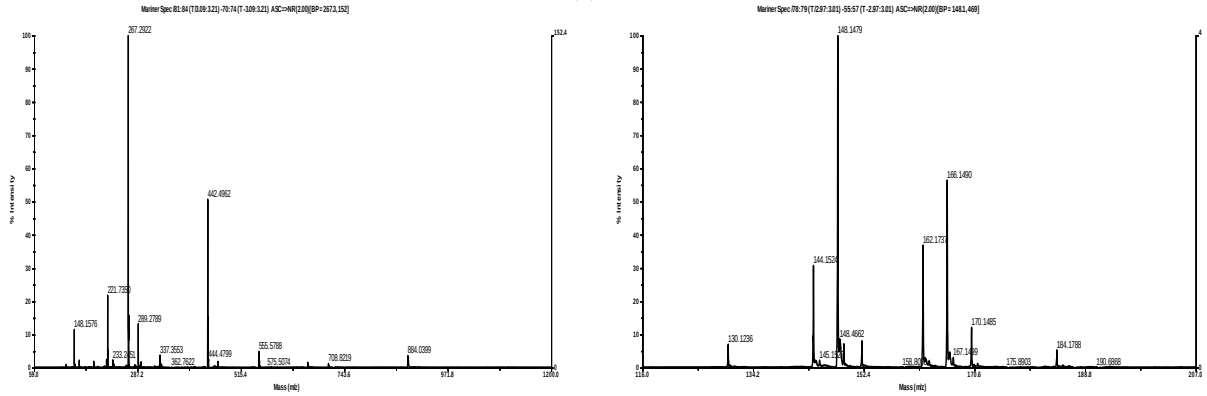
*) Each value represents an average from three trials.‡ SD values of folic acid (µg/mL) were in the range of ± 0.5–3.70 %.

Figure 5. Effects of fermentation time and the ratio of inoculum and isolates of *Rhizopussp.*and*S. cerevicae*on folic acid of fermented soybeans.



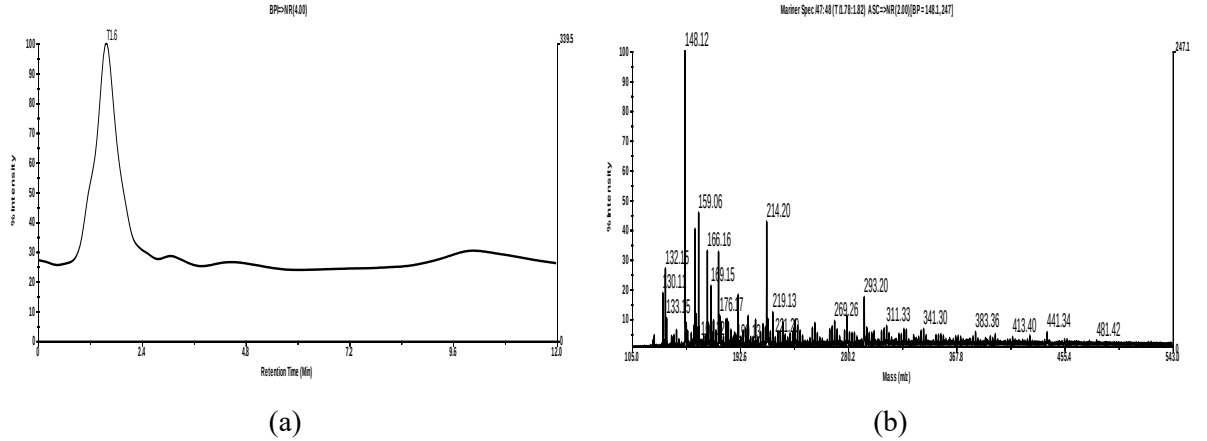
(a) (b)

Figure 6. Folic acid standard (a) and glutamic acid standard (b)



(a) (b)

Figure 7. Mass spectra of T 3.1 from standard chromatograms of folic acid (a) and T 3.0 from standard chromatograms of glutamic acid (b).



(a) (b)

Figure8. Chromatogram oligomer of amino acids as protein isolate soybean tempe with a retention time between 59-1200 min (a) and Mass spectra of T 1.6 from chromatogram soybean tempebetween 106 -543 m/z and optimum process conditions based on dissolved proteins and folate acids in inoculum ratio of *Rhizopussp*: *S. cereviceae* (1: 3), 72 hours (b).

Table 2. Identification of folic acid and glutamate acid compounds in soybean fermentation at the optimum condition with a combination of inoculum *Rhizopussp* and *S. cereviceae* (1: 3) for 72 hours.

No	Centroid Mass	Relative Inter (%)	Area	Chemical Names
1.	137.140306	4.02	87.13	4-aminobenzoid acid (PABA) $C_7H_7NO_2$
2.	145.156904	1.87	33.23	2-aminopentanedioateglutamate$C_5H_7NO_4^{-2}$
3.	147.137586	1.76	43.67	D-glutamic acid $C_5H_9NO_4$
4.	148.120132	100	1415.56	L-glutamate $C_5H_{10}NO_4^+$
5.	159.060508	45.42	828.54	Lithium L-glutamate $C_5H_8NNaO_4$
6.	163.167017	4.36	98.55	2-amino-4-hydroxypteridine
7.	169.112015	20.87	366.54	Natrium glutamate $C_5H_8NNaO_4$
8.	175.183459	32.36	670.39	L-Glutamic acid 5-ethyl ester $C_7H_{13}NO_4$
9.	176.169677	10.86	225.94	4-hydroxymethylglutamate $C_6H_{10}NO_5^-$
10.	181.182583	9.57	175.95	Diamonium L-glutamate $C_5H_{15}N_3O_4$
11.	182.165041	9.75	173.17	Monoamonium glutamate monohydrate $C_5H_{14}N_2O_5$
12.	185.171846	6.15	148.08	Potassium L-glutamate$C_5H_8KNO_4$
13.	187.158653	3.75	57.40	L-Glutamic acidmonosodium saltmonohydrate $C_5H_{10}NNaO_5$
14.	191.118699	17.88	332.51	L-Glutamic acid disodium salt$C_5H_7NNa_2O_4$
15.	207.184114	3.48	41.37	Pterin-carboxylic acid $C_7H_5N_5O_3$
16.	237.233275	9.48	197.25	L-Glutamicacid 5-benzyl ester $C_{12}H_{15}NO_4$
17.	264.169257	1.89	38.75	4-aminobenzoyl-glutamate $C_{12}H_{12}N_2O_5^{-2}$
18.	439.407602	2.70	45.12	5,8-Dideazafolic acid$C_{21}H_{21}N_5O_6$
19.	441.397255	5.28	107.96	Isofolic Acid$C_{19}H19N_7O_6$
20.	443.417706	2.49	49.73	(6S)-5,6,7,8-tetrahydrofolate dianion $C_{19}H_{21}N_7O_6^{-2}$
21	451.378534	2.19	34.33	Calcium N-(1-oxooctadecyl)-L-glutamate$C_{23}H_{41}CaNO_5$
22.	457.437005	2.96	46.75	5,10-methylenetetrahydrofolate $C_{20}H_{23}N_7O_6^{-2}$
23.	459.390297	2.22	37.52	5-methyl-tetrahydrofolate $C_{20}H_{25}N_7O_6$

4. Discussion

4.1. Chemical characteristic of soybean and visual observations of tempeh fermentation conditions with combination of Rhizopus sp. and yeast S. cereviceae

The compositions of soaked soybean and steamed soybean were various. These differences were influenced by several factors, such as pH, temperature, and cooking process. Steamed soybean presented low folic acid content due to longer period of cooking time.

Soaking process aimed to hydrate soybean and to naturally ferment lactic acid for obtaining the acidity which was needed for fungi growth for fermentation of soybean. Steaming process intended to hydrate the material and to soften the beans, as well as to inactivate phytase enzyme as a cause of rotten smell.

Fermentation involving yeast activity was reported to increase folic acid content in some fermented food products, although the detailed mechanism and the role of the yeast is unknown [17]. Yeast was also reported to have a role in the improvement of folic acid content in fermented soybeans into tempeh, for example: the activity *S. boulardii, G. candidum, Y. lipolytica,* and *A. pullulans*, can produce tempeh with better folic acid content in the presence of yeast [14]. Therefore, the role of yeast as a contributor of folic acid improvement in the fermentation of tempeh needs to be studied further. This research studied the development of tempeh inoculum of *Rhizopus sp*. combined with *yeast Sacharomyces cereviceae*. The soybeans fermentation was done at room temperature and 30 °C for 0–72 h to produce different composition and physical quality of tempeh. Visual appearances of soybeans without and with yeast in various ratios were shown in Figure 1.

4.2. Effects of fermentation conditions on the characteristics and compositions of soybean tempeh and concentrations of folic acid

4.2.1. Total Solids (%)

During the fermentation process, beans substrates experience distinctive changes, and one of them is transformation of its physical form from solid to semi-solid.Moreover, the increase of water content in the material is proportional with the transformation of complex components to the simple one. However, the water content in the substrates is inversely proportional to the total solids.

Figure 2 showed that total solid obtained after fermentation of soybean tempeh fluctuated, but the results were not significantly different during the fermentation time ($P>0.05$). It was shown that total solids value to the ratio of mold and yeast were significantly different during the fermentation time ($P<0.05$). The results of polynomials orthogonal test showed that the tendency of obtained results in soybean tempeh with the increase of mold and yeast ratio resulted in significantly different total solids ($P<0.05$) to the percentage of total solids, with the highest total solids were in the ratio of 1:2 which the total solids has degrees cubic equation of $Y = 0.0394x3 – 0.7093x2 + 4.2506x +37.435$, and determinant coefficient ($R2$) = 0.763, which means that the longer fermentation time affected to 76.30% of the total percentage of solids and the remaining 23.7% was influenced by other factors. From the equation, it was found that the optimal fermentation time was 72 hours with 49.30% ± 1.64 percentage of total solids.

Total solids were inversely proportional to the water content value, where the highest total solids or the lowest water content was obtained from soybean fermentation with mold to yeast ratio at 1: 2. It can be explained that the water in the materials was used for the growth of mold. As the number of yeast added increased, fermentation time was faster by utilizing the available water in the beans. Excessive amount of moisture inhibits the growth of mold. The reason is each mold has optimum Aw to grow. Hydration also provides Aw 0.99 which is optimal for mold to grow. In addition, the water content of the beans before fermentation influences the growth of the mold. During fermentation, mycelium will grow and bond on the soybeans [18]. Fermentation occurs in 18–48 h [19].

The increase of total solids occuring during fermentation was influenced by both the nature of material and fermentation temperature. Morever, it can be due to higher metabolism of proteolytic and amylolytic activity of inoculum sp. and yeast *S. cereviceae*, thus, greater amount of water are required. The increase of water content or total solids in tempeh can be due to moisture absorption from air and activity of microorganisms.

4.2.2. Dissolved Protein (mg/mL)

Dissolved proteins are an oligopeptides or amino acids that are easily absorbed by the digestive system. Fermentation time will increase modifications of chemical composition of soybean tempeh. The results of dissolved proteins seemed to fluctuate for four different inoculum ratios as a function of fermentation time, as shown in Figure 3.

It can be seen from the figure that the fermentation time and inoculum ratio of *Rhizopus sp.* and *S. cerevicae* provides the same patterns with sigmoid shaped curve in which the overall results affectedthe dissolved protein during fermentation at 30□C. Fermentation time showed significantly different total average value of dissolved proteins ($P<0.05$) versus each ratio of inoculum. However, inoculum ratio did not affect significantly on each fermentation time. The obtained tendency result of orthogonal polynomials in soybean tempeh with the increasing number of mold with yeast ratio resulted in significantly different ($P<0.05$) against the average dissolved protein. The obtained dissolved protein has cubical degree pattern with the equation $Y = -0.0102x3 + 0.1698x2 - 0.6112x + 0.8437$ and determinant coefficient ($R2$) = 0.8683, which means that longer fermentation time affected about 86.83% of the average dissolved protein and the remaining 13.17% influenced by other factors.From the equation, it was found that the optimal length of fermentation time was 72 h at the ratio of inoculum and yeast of 1:3, with an average dissolved protein value of 1.73 ± 0.02 mg/mL. Alteration of dissolved protein contents during fermentation occurred due to the role of enzymes produced by inoculum and yeast during the process. Protease enzyme was produced by both two types of microorganisms, which the protease enzyme breaks down the protein in soybeans into more simple compounds. *Rhizopus sp*. (a mixture of *R. oligosporus, R. stolonifer, R. oryzeae* and *R. arrhizus*) affects the proteolytic activity of the produced inoculum. This *Rhizopus sp*. inoculum mixture improves its ability to break down the protein peptide chain into a more simple chain. Increasing the number of yeast in to the mixure did not

show significant influences on the proteolytic activity produced in soybeans, however it resulted a better dissolve protein compared to the tempeh produced without yeast (*S. cereviceae*).

4.2.3. Nitrogen Amino (mg/mL)

As shown in Figure 4, the tendency results of fermentation rate on N-Amino production during fermentation of soybeans fluctuated, however, the obtained data showed that they are are not significantly different during the fermentation time ($P>0.05$). The average value of N-Amino to the ratio of mold and yeast seems to be significantly different during the fermentation time ($P<0.05$).

The results of orthogonal polynomials showed that tendency of results obtained in tempeh soybean with the increasing number of the ratio of mold with yeast yielded a significantly different ($P<0.05$) total solids to the average N-Amino, the average value of the highest are in the ratio of 1:1 with the equation $Y = -0.0096x5 + 0.2572x4 – 2.5056x3 + 10.809x2 – 19.408x + 12.171$ and determinant coefficient (R2) = 0.872, meaning that the longer the fermentation time gave an effect about 87.2% ± 0.05 of average N-amino and the remaining 23.7% influenced by other factors. From equation,it was found that the optimal length of fermentation time was 64 h with an average value of N-amino was 6,73 mg/mL.

The increase of N-amino was related to the proteolytic activities of *Rhizopus sp*. and yeast, where peptides that have not been broken down into amino acids, and further decomposed into free amino acids as well as deamination to form ammonia. Therefore, N-amino can be used as an indicator of acceptance through organoleptic from flavor and physical properties of tempeh. Well distributed and compact binding of mold (mycelia) growth produced amino acids with degradation results of produced enzyme from the mixture of mold inoculum and yeast, hence, it improves the digestibility of tempeh protein. This results agreed with the results of Kustyawati [14] that the yeast can grow together with *R. oligosporus*, and its growth may encourage the growth of mold on tempeh and change the appearance and flavor tempeh. Yeast is a part of fermented food microfloras, which their roles vary depending on their types. Generally, yeast contributes to the interaction between microorganisms, alters the textures and flavors of bio-sinthesized components [20]. Enzyme produced by the yeast is capable of hydrolyzing fats and proteins and producing aroma-precursor components, for example: volatile sulfur components (VSCs), and hydrolyzing α and β-casein which increase the level of amino acid [21,22].

4.2.4. Folic Acid (µg/mL)

Tendency results on the fermentation rate of folic acid yields during the fermentation time data showed that soybean tempeh was fluctuated, the obtained data during the fermentation time was significantly different ($P<0.05$). Same case for the average value of folic acid to the ratio of mold and yeast seem to be significantly different during the fermentation time ($P<0.05$), as shown in Figure 5.

The test of polynomials orthogonal tendency of the results obtained in tempeh soybean with increasing number of mold and yeast ratio provided the results of folic acid were significantly different ($P <0.05$) to the average of folic acid, the average value of the highest are in the ratio of 1:3 with fermentation time of 72 h with the equation of $Y = -0.0867x4 + 2.6584x3 – 24.656x2 + 80.574x + 257.93$ and the determinant coefficient (R2) = 0.703, which means that the longer fermentation time affected about 70.3% of the average N-amino and the remaining 30.7% influenced by other factors. From the equation, it was found that the optimal period of fermentation process was 72 h with an average value of folic acid amount of 381.63 ± 0.67mg/mL. The activity of mold and yeast may increase the folic acid content. These results were consistent with reports Murata et al. [11] that total folate of fermented boiled soybeans with *Rhizopus* increased four to five-fold with fermentation time of 48 h and extended to 72 h. The increase of total folate was probably due to the release of total folate by *Rhizopus* enzymes, which naturally presents or by synthesis in soybeans [23].

4.2.5. Folic acid identification by LC-MS

Identification of folic acid and glutamate acid compounds in soybean fermentation at the optimum condition with a combination of inoculum *Rhizopus sp* and *S. cereviceae* (1: 3) for 72 hours base on concentration dissolved protein and folic acid soyben tempe.

Folic acid or folasin is composed of 2-amino-4-hydroxy acid pteridine and p-amino benzoate which binds p-amino acid glutamate bezoat (PABG). Folic acid has empirical formula of C19H19N7O6 and molecular weight of 441.4 kDa. Folic acid is stable to bases, but it can be hydrolyzed with acid breaking down the side chains, thus, producing PAGB and p-terin-6-carboxylic acid. Folic acid is a heterocyclic compound with a structure pteorat acid conjugated with one or more L-glutamate which are connected through carbonyl group γ-amino acids. Folic acid has a residue of L-glutamic with the name of "pteroilglutamat acid". Folic acid can be reduced to H2 folate or tetrahydrofolate (H4 folate) which is the active form of co-enzyme vitamins [24].

Folic acid can be identified by standard folic acid and glutamic acid as shown in Figure 6a and 6b using LCMS for each type of tempehs (soybeans, green beans and red beans), based on the best process conditions for the dissolved protein results.

Figure 7a showed that folic acid standard is dominated by compounds with molecular weight of 442.5, 443.16, 443.51 and 444.48 Da. with intensities of 50.55, 8.35, 15 and 2.44%, respectively.In other words, folic acid could be assigned to this molecular weight. Figure 7b showed that the standard of glutamic acid is dominated by compounds with molecular weights of 148.15, 148.47 and 149.14 Da. with intensities of 100, 8.51 and 7%, respectively.In other words, it is possible that the glutamic acid is assigned to this molecular weight.

The results showed that the mass spectra of soybean tempeh obtained from inoculum ratio of *Rhizopus sp* inoculum with *S. cereviceae* (Figure 8) compared with the mass spectra of folic acid and glutamate standard (Figure 7a and 7b), were identified a number of 223 compounds at T 1.6, where eight compounds were considered as folic acid with a molecular weight of 137.14, 163.15, 207.18, 439.40, 441.39, 443.41, 457.43, and 459.39, with the relative intensities are 4.02% 4.30% 3.48%, 2.70%, 5.28% 2.49%, 2.96%, and 2.22% respectively, whereas identical compound as glutamic acid obtained from fifteen compounds with a molecular weight of 145.15, 147.13, 148.12, 159.06, 169.11, 175.18, 176.16, 181.18, 182.16, 185.17, 187.15, 191.12, 237.23 , 264.16 and 451.37, with the relative intensities are 1.87%, 1.76%, 100%, 45.42%, 20.87%, 32.36%, 10.86%, 9:57%, 9.75%, 6:15%, 3.75%, 17.88%, 9:48 %, 1.89%, and 223% respectively. The existence of folic acid in fermented soy beans are presented in Table 2.

5. Conclusion

The results showed that the interactions of fermentation time and inoculum ratio influenced the mold growth, overall appearances and compositions of tempeh. From the best process conditions obtainedbased on dissolved protein and folic acid content on soybean tempeh using inoculum mixture (*Rhizopus sp.* with yeast) at temperature of 30 °C for 72 h, the composition of dissolved protein, N-Amino, folic acid and total solids were 1.67 mg/mL, 4.48 mg/mL, 381.63 mg/mL, and 42.44%, respectively. Identification of folic acid as part of glutamic acid in soybean tempeh from fermentation at the optimum condition based on dissolved proteins showed that a number of 223 compounds at T 1.6 were identified, in which eight compounds were considered as folic acid and fifteen compounds are identical as glutamic acid. Tempeh inoculum development by combining inoculum *Rhizopus sp.* and yeast *S. cerevisiae* increases folic acid contents than only using *Rhizopus sp*. The hypothesis of our study was the addition of yeast could significantly increase folic acid content in fermented tempe.

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