International Proceedings of Chemical,
Biological & Environmental Engineering

Biology, Environment and Chemistry IV

IACSIT PRESS
http://www.iacsit.org.org/press.htm

2013 4th International Conference on Biology, Environment and Chemistry
IPCBEE vol.58 (2013) © (2013) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2013. V58. 7

Isolation and Characterization of Lactic Acid Bacteria from Indonesian Soybean Tempe

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Abstract. Tempe is a widely consumed Indonesian traditional food which is made from soybean through a fermentation process, mainly by *Rhizopus oligosporus*. Lactic acid bacteria (LAB) also involves in the process specifically in the soaking and fermentation steps. Isolation of the LAB from tempe was done at different stages of the tempe's production to examine for the occurrence of LAB. Morphological, physiological and biochemical characteristics were employed to identify LAB. 16 LAB were obtained and 13 LAB isolates were identified as Lactobacillus heterofermentative, one isolate, (S4 I) as Streptococcus non enterococci while the other 2 LAB isolates, (S4 A and S4 J) could not be identified. Hence this study showed that Lactobacillus heterofermentative bacteria is dominant in every stage of tempe's production.

Keywords: soybean, tempe, lactic acid bacteria, isolation, characterization

1. Introduction

Fermented food can be define as food substrate being employed by edible microorganisms with their enzymes function to hydrolyze the respective substrates along with desirable changes of flavours, aroma, and texture. Simply it defines fermented food as microbial initiated conversion of sugars into alcohol and lactic acid. Fermentation is an ancient preservation technique but still favoured to be use until now despites all the other new preservation techniques. Obvious factors contributing to the preferences of fermented food over unfermented food are it enhances the quality of food (flavour, aroma and texture), improved shelf life, increased nutritional value and safety [1]. This preservation technique had been applied to various type of raw material commonly legumes, fruits, vegetables, meat and fish. This study will focus on one of the famous fermented food known as tempe.

Tempe is an Indonesian traditional fermented food that is widely consumed in Malaysia. Tempe is define as compact, sliceable mass of cooked raw material being fully covered, penetrated and held together by dense non sporulated mycelium of *Rhizopus sp*. This food is made from soybeans (*Glycine max*), but can also be made from a variety of legumes and seeds as the main raw ingredient. Report by Hachmeister & Fung [2] covered the used of various legumes and cereals as raw material in tempe making. Shurtleff & Aoyagi, [3] reported that people who consume tempe in their daily basis were less susceptible to have any gastrointestinal problems. Many studies and reviews have been done regarding soybean tempe [4-7]. The concern of consumers demanding on healthy food is increasing rapidly nowadays. The occurence of lactic acid bacteria in tempe has been reported previously [5, 8]. Lactic acid bacteria has been known deliberating antimicrobial activity to inhibit the growth of pathogenic bacteria and may also play an improtant role in tempe fermentation. However, the number of local lactic acid bacteria (LAB) strains isolated from tempe is still scarce. As a result, isolation of LAB from local resources like tempe is necessary. The significant of this study is to make use of local fermented product (tempe) to its best use.

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2. Materials and methods

2.1. Sample collection

Samples at different stages of tempe production were obtained freshly from local producer in Bogor, Indonesia. The 4 samples collected for this study were; 1) soaking water, 2) soybean + inoculum (1st day), 3) soybean + inoculum (2nd day) and 4) tempe (3rd day). All samples were freshly analyzed on the same day they were obtained and were stored at frozen temperature. This study involved 3 stages which were; (1) isolation of LAB from different stages of tempe production, (2) purification of LAB, and (3) preliminary identification of LAB.

2.2. Isolation of LAB

10 ml of each sample were aseptically added into 90 ml of sterile KH_2PO_4 solution and was mixed thoroughly. Serial dilutions were performed and 1 ml aliquots of appropriate dilution were directly inoculated in duplicate on the de Man Rogosa Sharpe (MRS) agar (Oxoid Ltd.,Basingstoke, UK) using the pour plate method and were incubated for 48 hour at 37 ℃. The colonies were counted and total number of viable cells was reported as colony-forming units (CFUs). White small colonies was randomly selected and isolated on fresh MRS agar.

2.3. Purification

Single developed colony was picked from the plates and subculture to purify. Pure bacterial strains were obtained after successive transfer of individual colony in the plates and incubated for 24 hour at 37 ℃. Culture preservation was performed on all the isolates obtained. Fresh overnight cultures of each isolate were grown and stored in the freezer at -80 ℃ in 20% glycerol.

2.4. Preliminary identification of LAB

Identification of LAB was done based on morphology, physiology and biochemical characteristic based on Harrigan & MacCance [9]. Morphological observations of LAB with Gram staining were carried out. Gram-positive cell is marked with purple colour or blue and gram-negative with light red. Based on previous description, it is known that LAB is Gram-positive LAB with shaped rod or cocci. Observation of physiology and biochemical properties of LAB encompasses:

a) Catalase test were done using freshly prepared 3% hydrogen peroxide (H_2O_2). Bubble formation that occurs once mixed with a liquid suspension culture H_2O_2 indicates that the bacterium was catalyses positive.

b) Growth at different temperature were done using MRS broth (Oxoid Ltd.,Basingstoke, UK) where they were incubated for 7-14 days with a series of temperatures of 10 ℃, 45 ℃ and 37 ℃ as a control. The existence of growth was observed by the presence of turbidity in the tube.

c) For growth at 4% and 6.5 % salt concentrations, one drop of fresh isolated culture were inserted in MRS broth of these concentrations and incubated at temperature of 37 ℃ for 7-14 days. Turbidity declares the existence of growth.

d) Production of carbon dioxide (CO_2) from glucose was done using the Gibson's semi-solid tomato juice liquid media. A drop of fresh isolates culture was added then the melt agar was poured on it for about 2-3 cm to create anaerobic conditions. Incubation was carried out at 37 ℃ for 2-5 days. Heterofermentative lactic acid bacteria will produce gas that is marked by the outbreak of the agar, as opposed to homofermentative.

e) Dextran production from sucrose test was conducted to distinguish species of the genus *Leuconostoc*. Dextran formation is characterized by colony formation mukoid (mucus).

3. Results and discussion

3.1. Isolation and purification of LAB

From the isolation process, it was observed that the number of LAB present varies at different stages. The data is shown in **Table 1.** As the fermentation time increased, the number of LAB present also increased except for the second sample which is inoculums and soybean (day 1). This is because the soybean had just

been cooked for certain period of time thus killing most microorganisms. A study done by Moreno *et al.,* [5] also comes out with the similar pattern at this stage of tempe production.

Table 1: LAB during tempe fermentation

Stage	Number of LAB at different stage of tempe fermentation (CFU/ml)
Soaking water	9.5×10^{8}
Inoculum + soybean (day 1)	7.3×10^{6}
Inoculum + soybean (day 2)	1.8×10^{8}
Tempe (day 3)	2.6×10^{8}

After the purification process, 36 isolates were obtained. It was observed that 27 of them were bacteria, 8 yeast and 1 mould (**Table 2**). Isolation by Moreno *et al.,* [5] also indicates the presence of mould and yeast at different stages of tempe production although they were cultured on different media. Presence of mould in the final product was predicted since the mould was purposely introduced into the food as inoculum. **Table 2** shows that although yeasts and mould were present, the bacteria still dominate this fermented food.

Table 2: Isolation and purification

Source	Number of isolate	Bacteria	Yeast	Mould
Soaking water	5	5	-	-
Soybean + inoculums (day 1)	10	8	2	-
Soybean+ inoculums (day 2)	12	8	4	-
Tempe (day 3)	9	6	2	1

3.2. Preliminary identification of LAB

The Gram staining is a very crucial test as it will help to identify whether the isolates are LAB or not. With that any yeast, mould and gram negative bacteria are excluded from the next step of identification. Only gram positive, rod and cocci shape bacteria were chosen as they represent the LAB characteristic. With that, as many as 17 bacteria isolates were used for further study.

The next most important test is the catalase test. Only LAB is catalyst negative. With that, 1 positive catalyst isolate was eliminated from proceeding to the next test leaving as many as 16 isolates. Test using Gibson's semi solid tomato juice media were done to differentiate between heterofermentative and homofermentative isolates. From the test, only 2 isolates were homofermentative which are S4I and S4J while the rest were heterofermentative. As for the dextran test, it was observed that only 1 isolate which is S4J, formed the colony formation mukoid. As for the growth at different temperatures, it was observed that all isolates do not grow at chill temperature but all isolates grew well at 15℃ and 42℃. Hence, it can be concluded that all isolates were thermopiles because they can withstand high temperatures. For growth at different salt concentration, all isolates were able to grow at 4% NaCl but only 3 isolates which are S3I, S4D and S4K grew at 6.5% NaCl.

The data for the preliminary identification of LAB done based on morphological, physiology and biochemical characteristic is as shown in **Table 3**. The genus of LAB isolate was identified by comparing to the LAB identification table by Axxelson [10]. With that, 13 isolates were identified as *Lactobacillus* heterofermentative, 1 isolate of S4I as *Streptococcus* non enterococci while the other 2 isolates of S4A and S4J cannot be identified. Study done by Moreno *et al.,* [5], successfully isolated *E. faecium* from the final product of tempe (7 day old). Isolation done by Kormin *et al.,* [8] on the other hand, identified *L. plantarum* BS2 from tempe having inhibitory activity. *Lactobacillus spp., Weisella spp*, and *Pediococcus spp* were the LAB isolated from the similar fermented soybean product known as douchi in Yunnan [11].

From this study it was observed that the highest number of LAB is in the soaking water. This data was supported by Mulyowidarso *et al*., [12] who reported that it was due to increase in organic acid. The LAB was important in the first stage as they will reduce the pH of soaking water and hence suppress the growth of pathogenic bacteria. Numerous studies showed that the occurrence of LAB in tempe can inhibit the growth of potential pathogenic bacteria [13-18].

Table 3: Preliminary identification for LAB identification

Source	Code	Gram	Shape	Catalyst	CO_2	Dextran	Temperature (℃)			Salt concentration (%)		Identified as
							5	15	42	4	6.5	
Soaking water	S1A	+	Rod	_	+	_	_	+	+	+	_	Lactobacillus heterofermentative
Inoculums + soybean (day 1)	S2G, S2 H	+	Rod	_	+	_	_	+	+	+	_	Lactobacillus heterofermentative
Inoculums + soybean (day 2)	S3D, S3 G, S3K, S3 M, S3J S3 N	+	Rod	_	+	_	_	+	+	+	_	Lactobacillus heterofermentative
	S3I	+	Rod	_	+	_	_	+	+	+	+	Lactobacillus heterofermentative
Tempe (day 3)	S4A	+	Cocci	_	+	_	_	+	+	+	_	Not identified
	S4C	+	Rod	_	+	_	_	+	+	+	_	Lactobacillus heterofermentative
	S4D, S4 K	+	Rod	_	+	_	_	+	+	+	+	Lactobacillus heterofermentative
	S4I	+	Cocci	_	_	_	_	+	+	+	_	Streptococcus non enterococci
	S4J	+	Cocci	_	_	+	_	+	+	+	_	Not identified

+ indicates production of CO_2 / mukoid/ turbidity

- Indicates no production of CO_2 / no mukoid/no turbidity

4. Conclusion

In this study, LAB has been successfully isolated from different stages of tempe production. Highest occurrence of LAB was discovered in the soaking water. Out of 16 LAB, 13 LAB were identified as *Lactobacillus* heterofermentative, 1 isolate of S4I as *Streptococcus* non enterococci while the other 2 isolates of S4A and S4J could not be identified.

5. Acknowledgements

This study was supported by the collaboration of Universiti Teknologi MARA (UiTM), Malaysia with Institut Pertanian Bogor (IPB), Indonesia under Malaysia-Indonesia-Thailand (MIT) student exchange program and funded by the Research Cluster Grant (600-RMI/ DANA 5/3/CG (18/2012)).

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