

Article

Development of Salt-Tolerant *Rhizopus oligosporus* for Tempeh Production

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<ABSTRACT>

Tempeh is Southeast Asian traditional food, and is salt-free fermented soybean food made using *Rhizopus oligosporus*. We have been trying up to now to establish a stable manufacturing method, and to get the right taste and flavor that would be favorable to Japanese taste, aiming at making tempeh widely available in the Japanese market. As a part of this purpose, we tried to develop a salt tolerant strain of *R. oligosporus* which is essential to add moderate saltiness (around 1%) to the raw materials of soybean before fermentation. The method of the strain development was to give salt-tolerance to a strain by repeating inoculation and cultivation of the cell body from the potato-dextrose medium containing either 1% or 2% salt in which the strain was grown, onto the fresh medium containing the same amount of salt. As a result, we succeeded in the development of a 1% salt-tolerant strain so that tempeh production can be carried out the same way as the conventional method.

Keywords: tempeh, *Rhizopus oligosporus*, development, salt-tolerance

INTRODUCTION

Tempeh is Indonesian traditional salt-free fermented food to process soybeans as raw materials with the use of a mold (*Rhizopus oligosporus*)¹⁾. Tempeh is full of nutritive value, and a lot of functional materials are included in it²⁾. There is not the mucilage substance and there are not the unique fermentation smell and taste such as the Japanese natto. A soft texture such as the cheese and plain taste are characteristics of tempeh. In the early 1990s, the introduction to the Japanese market of the tempeh was tried for the first time by some groups of food industry and a research group in Kyushu University, but the result was not desirable. Although the same trial has been conducted for several times, the result was in

vain. There is the natto, the soybean fermented food which is traditional in Japan, and the conclusion that food industry gave as the reason why tempeh does not take root in the Japanese market is that there is not a characteristic superior to natto for Japanese consumers to tempeh³⁾. In other words, the tempeh has defects following than natto. 1) tempeh is expensive, 2) tempeh is too simple in flavor and taste for eating as it is without cooking, 3) it is popular to eat tempeh after being fried with breading, but a health image is spoiled remarkably by oil absorption in the fried tempeh. These factors become the neck for consumption expansion of the tempeh into Japan. One of the means to improve the taste of tempeh is to eat it with some seasonings or salt. It is well known fact that there is the synergistic action to taste in

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the usage of seasonings and salt. With respect to giving salt to tempeh, it is desirable for raw materials soybean to be given saltiness at a stage of the fermentation to avoid an excessive salt intake to human body. Therefore we tried the development of the tempeh which could be tolerable to salt at the fermentation stage of tempeh production in this study.

MATERIALS AND METHODS

Strains

Rhizopus oligosporus NBRC8631 (synonym of NRRL2710) which is an experimental strain superior in flavor and taste of the tempeh was used in this study. This strain was isolated and characterized by K.H. Steinkraus⁴⁾.

Determination of salt content of soybeans dipped in salt solution

Salt solution was prepared 0 to 10% by every 1% aliquot. 2ml of salt solution and two grains of soybean (HIGHPRO, USA) were put into a test tube, and placed at 25°C for 24hrs. The soybean was triturated with POLY TRON PT10-35 (Kinematica AG., Switzerland) at power 5 for 5min to get smooth suspension. Sample solution was obtained as supernatant after centrifuged at 5,000 rpm for 5min at room temperature in a microtube. The supernatant was used for the determination of salt concentration with a commercial salimeter (PAL-SALT, ATAGO CO. LTD., Tokyo).

Growth determination of *R. oligosporus* NBRC8631

R. oligosporus NBRC8631 was cultured on the potato-dextrose agar plate (Merck KGaA, Germany) until spore grew co-fluently in the sterilized plastic plate (9cm in diameter). 1ml of sterilized water was added into the plate and the spore suspension was moved into a sterilized tube. This spore suspension was called as the spore suspension. The spore suspension was always freshly made for each experiment.

The number of spores in the spore suspension was approximately 1×10^9 /ml. A paper disc (for the use of antibiotic inspection, ϕ 8mm, Advantec, Tokyo) was placed in the center of a potato-dextrose agar plate. 30 μ l of the spore suspension was let soak into a paper disc. The culture plate was left to stand at 25°C in an incubator. The circular colony was formed with the *R. oligosporus* spawn, and the diameter of four diagonal directions with a ruler was measured at every 24 hours and the mean value was regarded as the degree of growth.

Development of salt-tolerant strain

R. oligosporus NBRC8631 was cultured on the potato-dextrose agar plate containing 1% or 2% salt for one week. It should be noted that development of salt-tolerant strain was carried out both in 1% and 2% of salt concentration in the medium each. A small amount of spores was taken with a sterilized and disposable plastic loop and planted onto a new agar plate containing the same amount of salt. Ten times of same operation was repeated and salt tolerance was given to the experimental strain. The degree of salt-tolerance was detected by means of the colony diameter grown on salty agar plate mentioned as the previous section.

Preparation of tempeh in a test tube and a plastic bag

The procedure was followed as described³⁾. 40g of soybean was placed in a beaker and 200ml of 0.5% sodium acetate solution was added. After 18hrs at 25°C, the hull was peeled manually and moisture was dropped off with a colander for five minutes. Then, 200ml of 0.5% sodium acetate solution was added to the soybean, and then boiled for 5min. After boiling was finished, moisture was dropped off with a colander for 5min and centrifuged with a spin-drier for 5min at 1,000 rpm (Hitachi PS-H45L, Tokyo). 100 μ l of tempeh starter suspension was added to the spin-dried soybean, mixed well in a plastic bag, and then put into a fermentation plastic bag (5 \times 7cm size and 40 pinholes on one

side). The fermentation bag was fixed with a flat board evenly and was fermented at 35°C for 20hrs.

RESULTS AND DISCUSSION

Target of strain development

The target concentration of salt-tolerance for the strain development was considered at first. It is said generally that the salt concentration favored by Japanese people is 0.9% (w/v) which is body fluid levels that is sodium chloride. Thus, it is desirable to design the salt content of food between 0.8-1.1% because there is the good balance between salt and other food compositions. In a process to dip raw soybeans in liquid overnight, saltiness can be added to the soybean. On a condition same as the process of real manufacturing of tempeh production^{3,5)}, the relation of salt content of the dipping liquid and the salt content of the dipped soybean overnight was examined, and the result was shown in Figure 1. After performing the sensory test of the analytical type in five experimenters, it was shown that 1.02% salt content that was acquired

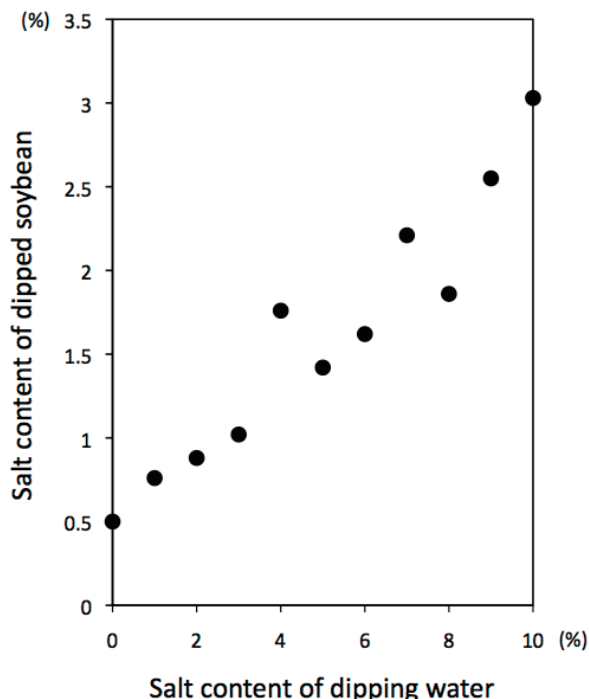


Figure 1. Relation of salt content between dipping water and dipped soybean.

by soybeans dipped in 3% salt water overnight was felt most deliciously. Therefore it was decided to develop it with the goal of giving 1% salt-tolerance to *R. oligosporus* in this study.

Figure 2 shows the representative appearance of the typical change in growth of *R. oligosporus* NBRC8631 which was grown on the nutrient agar medium containing variable content of salt. Figure 3 shows the growth status affected by variable salt concentration graphically. It was observed that the growth of *R. oligosporus* became slow as the salt content increased. It was shown that the growth rate of *R. oligosporus* declined conspicuously at around 0.6% concentration of salt in the medium. If the growth of *R. oligosporus* becomes slow, the propagation of various germs happens during tempeh production, and tempeh might decay⁶⁾. The spoilage of tempeh by unnecessary germs should be avoided absolutely from the viewpoint

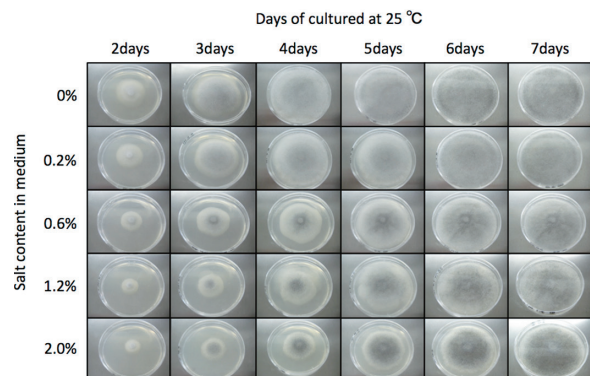


Figure 2. Daily change in growth for *R. oligosporus* NBRC8631 when salt content of medium was varied. Representative data is shown here.

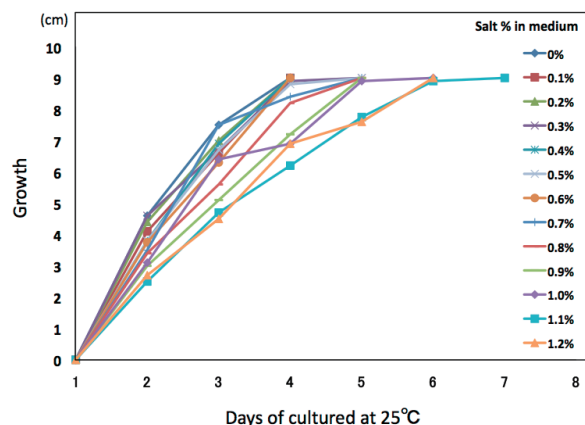


Figure 3. Growth curve of *R. oligosporus* NBRC8631 on potato-dextrose medium when salt content was varied from 0% to 1.2% by every 0.1% aliquot each.

of food poisoning prevention. Therefore, the specific target of the strain development in this study is that the growth rate of *R. oligosporus* won't slow down and also sporulation which worsen the appearance and taste of tempeh won't occur in 1% of salt content.

Evaluation of the acquired salt-tolerance

About *R. oligosporus* which acquired salt-tolerance in the nutrient medium of 2% salt, the examined salt-tolerance was shown in Figure 4. And about *R. oligosporus* which acquired salt-tolerance in the nutrient medium of 1% salt, the examined salt-tolerance was shown in Figure 5. It was clearly shown that the strain developed in a 1% salt medium acquired stronger salt-tolerance when both strain was compared for some reason. Probably, as for the 2% salt content medium, it was thought that the breeding condition was too severe to get salt-tolerance.

Figure 5 shows that the strain developed in the 1% salt medium acquired about 1% salt-tolerance. There was no difference observed between the original strain and the salt-tolerant strain for the state of sporulation (data not shown). Then the tempeh was produced with salted soybean of 1% salt content using 1% salt-tolerant strain and the appearance of the product was compared with the tempeh made

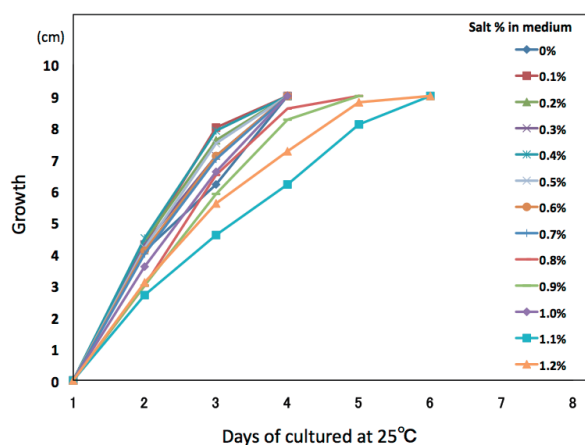


Figure 4. Growth curve of salt-tolerant *R. oligosporus* NBRC8631 developed with 2% salt content in medium. The growth was detected on potato-dextrose agar plate, when salt content was varied from 0% to 1.2% by every 0.1% aliquot each.

with non-salted soybean using original strain. Figure 6 shows appearances of both tempeh produced. There was no difference observed in the state of fermentation and sporulation.

From the result mentioned above, it was confirmed that we succeeded to develop the 1% salt-tolerant strain of *R. oligosporus*. For further study, we aim at the development of the tempeh of the taste that matches to Japanese taste, by examining seasoning the dipping liquid using this 1% salt-tolerant strain.

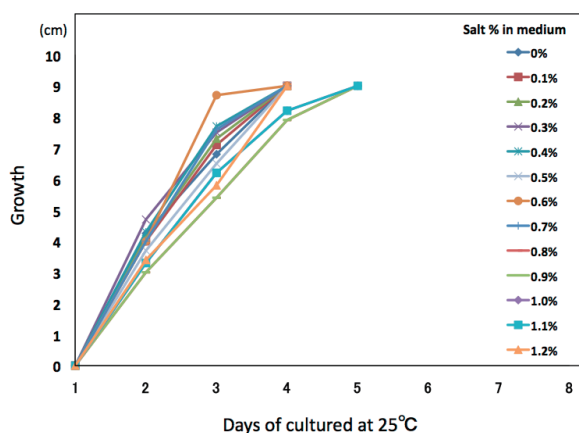


Figure 5. Growth curve of salt-tolerant *R. oligosporus* NBRC8631 developed with 1% salt content in medium. The growth was detected on potato-dextrose agar plate, when salt content was varied from 0% to 1.2% by every 0.1% aliquot each.



Figure 6. Comparison of tempeh produced with original (= "Salt-free") and salt-tolerant (= "Salted") *R. oligosporus* NBRC8631. Raw soybean of "Salted" tempeh was dipped in 3% salt water when the salt content of the dipped soybean became approximately 1%.

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食塩耐性のあるテンペ菌 *Rhizopus oligosporus* の育種開発

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＜要 旨＞

テンペは東南アジアの伝統食品であり、*Rhizopus oligosporus*を用いて作る無塩大豆発酵食品である。これまで我々は、テンペを日本市場に普及させるために、安定した製造方法の検討や、日本人の嗜好に合った風香味を得るための検討を試みてきた。今回その一環として、原料大豆に適度な塩味(1%程度)をつけて発酵を行うために必要な、テンペ菌 *R. oligosporus*の食塩耐性株の獲得を目指して育種開発を行った。育種の方法は、1%もしくは2%の食塩を添加したポテト・デキストロース寒天培地に生育した菌体を、何度も植え継ぎを繰り返すことで、その菌株に食塩耐性を付与させることを試みた。結果として、テンペの従来の製造において問題がない1%食塩耐性株の育種に成功した。

キーワード：テンペ、*Rhizopus oligosporus*、育種、食塩耐性