# Isolation and characterization of lactic acid bacteria starter for preparation of fermented Khanom-jeen

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## Abstract

Khanom-jeen (fermented rice noodles) is a traditional fermented food of Thailand since ancient times. Generally, flavor and taste from khanom-jeen occur as by-products from bacterial natural fermentation. Lactic acid bacteria (LABs) are microorganisms that play important roles in khanom-jeen process. In order to maintain their consistency of product, therefore, homofermentative LABs were screened and selected for use as a starter culture. LABs from this study were isolated from broken rice, fermented rice flour and rice noodle. The 268 isolates from 50 samples were Gram-positive bacteria, could not produce catalase enzyme and gas. LABs isolates were tested for their properties basis on % lactic acid and exopolysaccharides production including their growth in litmus milk and their ability to hydrolyse protein and starch. Our LABs were identified after compiling the results from api 50 ®CHL test kit. The selected *Lactobacillus crispatus* FF206, a high lactic acid and amylase producing and *Lactobacillus paracasei* ssp. *paracasei* R190, a high protease but not bioamine producing LABs will be used as starter culture(s) for preparing khanom-jeen in the future.

**Keywords:** hanom-jeen, lactic acid bacteria, homofermentative LABs

## Introduction

Rice (*Oryza sativa*) is one of the most important cereals consumed as a staple food in Asia. Aside from cooking, the rice can be processed differently to various products. The annual growth rate of Thai processed rice products has increased continuously until now with the rate of 2.61 percent per year. Khanom-jeen is a traditional fermented rice noodle widely taken and popular among consumers from all regions of Thailand. There are 2 kinds of Khanom-jeen in the market which are non-fermented and fermented ones. However, Thai consumers prefer eating fermented rice noodles because they confer unique flavor and textural characteristics (Oupathumpanont et al., 2008). Currently, commercial production of fermented rice noodles range from household to industrial. However, irregular qualities; for example, color, flavor, taste and many undesirable properties are commonly found in the rice noodles with natural fermentation from different batches. Therefore, quality control during process has to be concerned seriously. During fermentation, lactic acid bacteria play important roles in every steps of Khanom-jeen preparation. Lactic acid produced by lactic acid bacteria improves the food quality through the development of flavor, enhancement of the nutritional value, extension of shelf life, provision of organoleptic properties and removing toxin or antinutritional factors from food products (Rollan et al., 2010). In addition, it prevents the growth of undesirable microorganisms. Moreover, fermentation increases a higher value added of product and it has been accepted that these products contribute in improving human health (Ray and Daeschel, 1992; Hutkins, 2006).

Lactic acid bacteria are generally recognized as safe (GRAS status) and play an important role in food. There are a group of related bacteria that produce lactic acid as a major metabolic product. Lactic acid bacteria are Gram-positive, non-spore forming and lack of cytochrome. They are fastidious microorganisms that require high nutrients for growth. Generally, LABs are found in nature such as dairy products, grain products, meat products including normal flora of humans. Lactic acid bacteria are divided into three groups based on lactic acid fermentation. Firstly, obligate homofermentative bacteria ferment glucose into lactic acid over 85 percent by the Embden-Meyerhof pathway. Secondly, facultative heterofermentative bacteria can ferment glucose to lactic acid by the Embden-Meyerhof pathway or pentose to lactic acid and acetic acid by phosphoketolase pathway. Lastly, obligate heterofermentative bacteria ferment either glucose or pentose to lactic acid only 50 percent, acetic acid (ethanol), and $CO_2$ by phosphogluconate pathway (Whitman, 2009).

By using broken rice mimic rice noodle for fermented Khanom-jeen preparation, it was found that *Lactobacillus* sp. and *Streptococcus* sp. could increase the acid from 0.8 to 1.6%, while the pH decreased to 3.8 to 3.9 in the last 18 hours of fermentation (Kraidej et al., 1977). Later in 2012 (Leamdum et al., 2012) good starch-degrading LABs, a total of 63 isolates were detected but only one isolate with excellent degrading ability on starch-yeast-peptone medium was found. At the same time (Abubakr et al., 2012), LABs isolated from 12 kinds of fresh fruits were demonstrated to perform proteolytic activity on skim milk agar with the clear zone greater than 6 mm.

In this study, therefore, in order to prepare for good quality and consistency fermented Khanom-jeen products, starter culture (LABs) with high starch and protein degrading activities and lactic acid producing were screened and isolated. Raw materials used for preparing fermented Khanom-jeen as well as ready to eat fermented Khanom-jeen from several sources were conducted as samples for LAB isolation.

**Methodology**

Isolation of Lactic acid bacteria

A 25 g of each sample (broken rice, fermented flour and fermented rice noodles) was taken aseptically and transferred to stomacher bags, with 225 ml of buffer peptone water and then homogenized for two minutes by Stomacher (Masticator, BEC-Thai, Thailand). Further ten-fold dilutions were made and then 100 µl of each dilution was spread on de Man, Rogosa, Sharpe (MRS) agar (add 0.04% Bromocresol purple) coupled with standard plate count agar. All chemicals and media are analytical grade purchased from Difco (Difco Laboratory, USA).

Selected colonies were tested for Gram staining, string test, cell morphology, and catalase with 3%$H_2O_2$. After that individual LAB cultured in MRS broth containing durham tube and litmus milk medium was observed for gas production and changes in medium. In addition, detection of histamine decarboxylase broth with histamine as precursor of biogenic amine (BA) was examined. Moreover, EPS production was determined using with sucrose gelatin agar (SGA). LABs were kept in MRS broth supplemented with 20% glycerol at -20$^{o}$C for use during this study.

Determination of pH and titratable acidity

Supernatant of individual LAB cultured in MRS broth for 24 h after centrifugation at 10,000 g 15 min was used for pH measurement and acid content. One ml of supernatant was mixed with 10 ml distilled water and then determined for the acid productivity by a titration method (AOAC, 2000).

Detection of proteolytic activity and starch hydrolysis

All LABs were restreaked on MRS agar for 24-48 h. Heavy inoculum of LAB was streaked on skim milk agar and MRS-starch agar then incubated at 37°C for 24-48 h. Clear zone surrounding colony on skim milk agar while MRS-starch agar topped with iodine solution before measurements was observed. The average diameter (mm) of clear zone minus with size of colony was recorded. *Bacillus cereus* and *Escherichia coli* were used as positive and negative controls, consecutively.

Identification of LAB

The LABs with the properties of producing homofermentative lactic acid, amylase, protease and exopolysaccharide but not producing BA were selected for use in this study. Individual LAB was cultured in MRS broth at 37ºC for 24 h and restreaked on agar for pure colonies. Cell suspension was prepared and added into various kinds of biochemical tubes. These LABs were later identified using api 50®CHL test strips (bioMe´rieux, France) and conventional techniques.

**Results**

Characteristics of selected LABs

LABs from a total of 50 samples from fermented rice noodle (n=23), fermented flour (n=11), broken rice (n=14) and tapioca starch (n=2) were isolated. All isolates changed a color from purple to yellow on MRS agar, catalase-negative, string test negative and Gram positive. Four hundred eighty-six presumptive LABs were isolated from all samples. Furthermore, non-producing gas homofermentative LABs of 268 isolates (55.14%) were found. The highest polysaccharide-producing isolates are FF17 and FF281 whereas R190 was not produced BA. Characteristics of selected LABs were indicated in Table1.

Lactic acid production

A total of 56 LABs produced lactic acid (%) at the range of 1.2%-2.7%. The highest of lactic acid (%) was found in FF213 (2.17%) with the pH decrease from 6.5 to 3.73 within 24 h. Figure 1 shows the lactic acid (%) and pH from selected LABs.

**Table1** Characteristics of selected LABs from various samples.

| LAB isolates | Type of samples | Counts (CFU/ml) | | Gram staining | BA | Polysaccharide production | Litmus milk medium |
|---|---|---|---|---|---|---|---|
| | | Total bacteria | Total LAB | | | | |
| **FF14** | Fermented flour (Phayao) | $1.4x10^6 \pm 13.9$ | $1.5x10^6 \pm 13.6$ | +, rod | + | - | Purple, Not change |
| **FF17** | Fermented flour (Phayao | $1.4x10^6 \pm 13.9$ | $1.5x10^6 \pm 13.6$ | +, rod | + | + | White , Not Curd |
| **RF114** | Broken rice fermentation | $9.6x10^8 \pm 20.0$ | $1.8x10^9 \pm 18.9$ | +, cocci | + | - | Purple Top, White Bottom, Curd |
| **FF127** | Fermented flour (Phayao) | $1.4x10^6 \pm 13.9$ | $1.5x10^6 \pm 13.6$ | +, rod | + | - | Purple, Not change |
| **R190** | Broken Thai jasmine rice | $1.3x10^5 \pm 8.9$ | $1.7x10^5 \pm 27.0$ | +, cocci | - | - | Purple Top, White Bottom, Curd |
| **FF206** | Fermented flour (Kanchanaburi) | $1.2x10^8 \pm 192.2$ | $1.2x10^8 \pm 9.0$ | +, rod | + | - | Purple, Not change |
| **FF213** | Fermented flour (Kanchanaburi) | $1.2x10^8 \pm 192.2$ | $1.2x10^8 \pm 9.0$ | +, rod | + | + | Pink Top, White Bottom, Curd |
| **GF215** | Fermented glutinous flour | $2.2x10^8 \pm 118.9$ | $9.7x10^7 \pm 23.5$ | +, cocci | + | + | Pink Top, White Bottom, Curd |
| **FF280** | Fermented flour (Nonthaburi) | $9.7x10^6 \pm 18.4$ | $9.1x10^7 \pm 8.1$ | +, rod | + | + | Purple Top, White Bottom, Not Curd |
| **FF281** | Fermented flour (Nonthaburi) | $9.7x10^6 \pm 18.4$ | $9.1x10^7 \pm 8.1$ | +, rod | + | + | Purple Top, White Bottom, Not Curd |

- = negative; + = positive

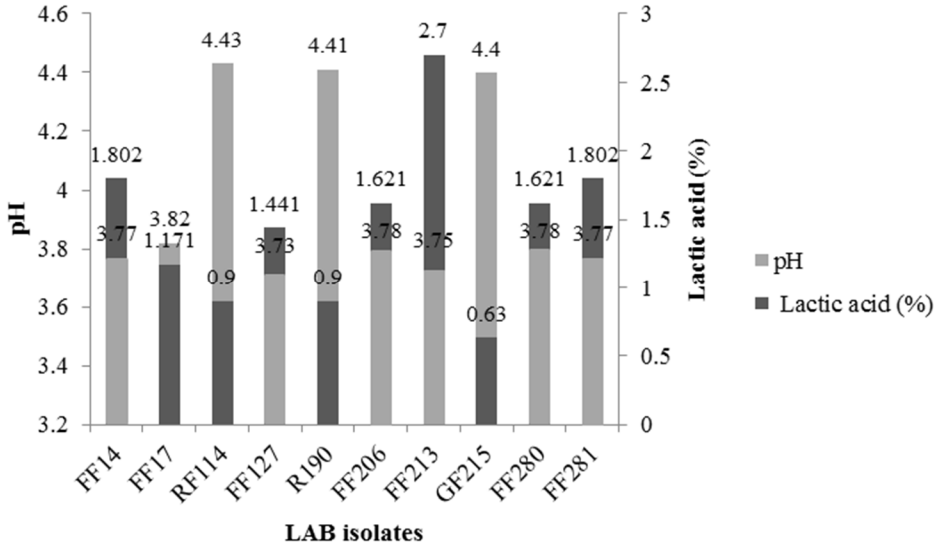


**Figure 1:** Lactic acid (%) and pH of the selected LABs after cultured in MRS broth for 24 hours.

Protein and starch hydrolysis

Figure 2 depicted protease activity and starch hydrolysis from selected LABs. RF114 and R 190 showed strong protein hydrolysis with the clear zone of 8.67±1.53 mm and 8.67±0.58 mm, respectively. In addition, the highest amylase activities with the clear zone of 11.67±0.58 mm was found in FF127. Protease and amylase activity on skim milk agar and MRS-starch agar, respectively were demonstrated as a zone surrounding LAB in Figure 3.

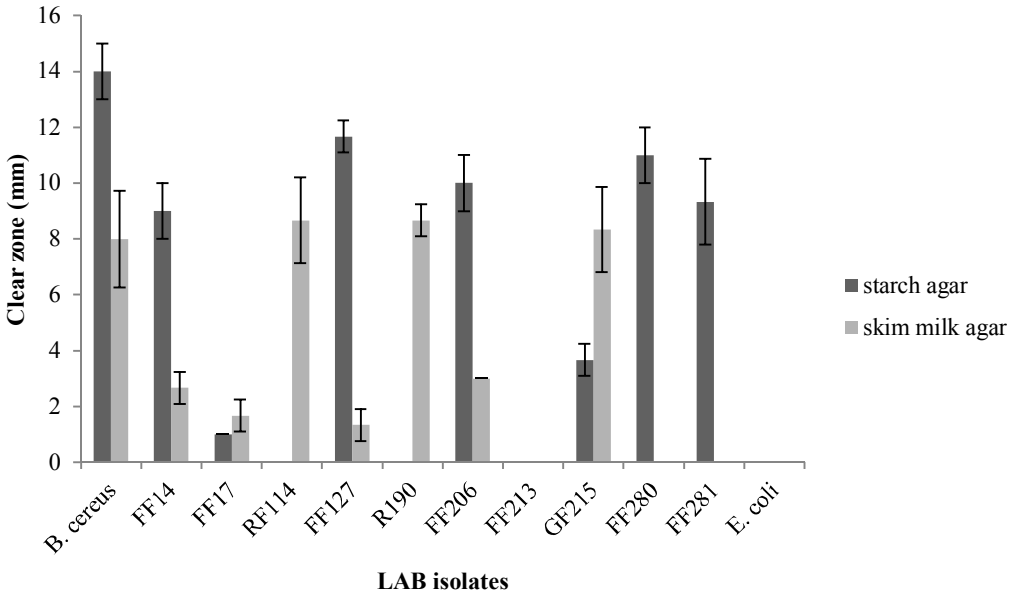


**Figure 2:** Comparison of clear zone (mm) from protease activity and starch hydrolysis of selected LABs.

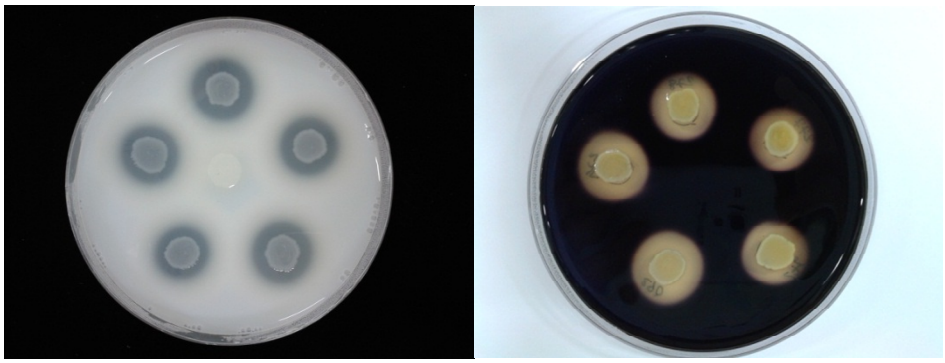

**Figure 3:** Plate assays for detection of protease and amylase activity of selected LABs on skim milk agar (left), and MRS-starch agar (right) which individual diameter was revealed after topped with iodine solution.

Identification of selected LABs

Selected LABs were identified using api 50 ®CHL test strips and indicated in Table 2.

**Table 2** Identification of selected LABs

| Code | Species identified | %Identity |
|---|---|---|
| FF14 | *Lactobacillus delbrueckii ssp delbrueckii* | 74.5 |
| FF17 | *Lactobacillus curvatus ssp curvatas* | 99.9 |
| RF114 | *Lactobacillus paracasei ssp paracasei* 1 | 92.8 |
| FF127 | *Lactobacillus delbrueckii ssp delbrueckii* | 81.5 |
| R190 | *Lactobacillus paracasei ssp paracasei 3* | 88.5 |
| FF206 | *Lactobacillus crispatus* | 97.9 |
| FF213 | *Lactobacillus paracasei ssp paracasei* 3 | 91.1 |
| GF215 | *Lactococcus lactis ssp lactis* | 70.9 |
| FF280 | *Lactobacillus paracasei ssp paracasei* 3 | 94.7 |
| FF281 | *Lactobacillus curvatus ssp curvatas* | 99.6 |

## Discussion

Homofermentative LABs are our aim for use as a fermented Khanom-jeen starter culture. Because Homofermentative LABs were produce lactic acid more than 85% as a preservative in many food products (Fossi et al., 2013). Taste and smell of lactic acid are better than other acids. FF14, FF127, R190 and GF215 with % identity lower than 90. Their 16S rDNAs will later be sequenced and reclassified. Our selected LABs represent the different features but they have potentials for use as a starter in Khanom-jeen fermentation. Mono-LAB culture may not have desired properties completely. Therefore, mixed LABs may be required to complete all features. Three LABs (*Lactobacillus plantarum* PD110, *L. cellobiosus* RE33 and *Leuconostoc lactis* PD128) isolated from fermented broken rice were previously isolated. Only individual isolate was tested and claimed to possess potentials for use as a starter for Khanom-jeen preparation (Sribuathong et al., 2005). In this study *Lactobacillus crispatus* FF206, a high lactic acid and amylase producing and *Lactobacillus paracasei ssp paracasei* R190, a high protease but not bioamine producing LABs were selected. In the future, these LABs will be used as mixed starter cultures for preparing fermented Khanom-jeen by following the procedures of commercial process.

## Conclusion

A total of 268 homofermentative LABs were isolated from broken rice, fermented flour and fermented rice noodles from various sources of Thailand. Bioamine (histamine) and exopolysaccharide producing LABs among our isolates were 96.64% and 2.24%, respectively. The highest lactic acid (2.17%) and pH of 3.73 was found in FF213. Only 45 isolates (16.79%) and 88 isolates (32.84%) produce protease and amylase, consecutively. FF206 is the best amylase producer whereas RF114 and R190 do for protease.

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