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Isolation and selection of *Lactiplantibacillus plantarum* CLT-L01 (TISTR P088) from traditional fermented vegetables for solid-state fermentation of soybean meal (SBM)

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ABSTRACT

Lactic acid bacteria are widely used as beneficial microorganisms in fermentation processes for animal feed production. In soybean meal, which is an important protein source in animal feed, lactic acid bacteria fermentation can improve nutritional quality by promoting acidification and reducing antinutritional factors. This study aimed to isolate lactic acid bacteria from traditional fermented vegetables and evaluate their potential as starter cultures for the solid-state fermentation of soybean meal. Traditional fermented vegetables were selected as the isolation source because they are locally available, inexpensive, and rich in naturally occurring lactic acid bacteria. Nine acid-producing bacterial isolates were obtained from traditional fermented vegetable samples using MRS agar supplemented with calcium carbonate. The isolates were preliminarily characterized by Gram staining and catalase testing. Six isolates, namely CLT-35, CLT-51, CLT-33, CLT-52, CLT-53, and CLT-L01, were selected for the evaluation of acid production efficiency in MRS broth. Among them, CLT-L01 showed the fastest acid production, with the pH decreasing to 3.21 ± 0.02 after 24 h of incubation. Therefore, CLT-L01 was selected for further evaluation in solid-state soybean meal fermentation. The results showed that soybean meal inoculated with CLT-L01 exhibited a rapid increase in lactic acid content, exceeding 10% after 96 h of fermentation. The highest lactic acid content was 11.13 ± 0.06% at 127 h, whereas the uninoculated control reached only 2.43 ± 0.12% at 151 h. These findings indicate that CLT-L01 enhanced acid production and improved fermentation efficiency compared with natural fermentation. Molecular identification based on 16S rDNA sequencing revealed that CLT-L01 showed more than 99% sequence similarity to the reference strain *Lactiplantibacillus plantarum*. Overall, CLT-L01 has potential as a starter culture for promoting lactic acid production and acidification during soybean meal fermentation.

Keywords: Lactiplantibacillus plantarum, Lactic acid bacteria, Solid-state fermentation, 16S rDNA

INTRODUCTION

Soybean meal (SBM) is a widely used plant protein ingredient in animal feed because of its high protein content, availability, and relatively low cost. However, its nutritional value can be limited by antinutritional factors (ANFs), such as trypsin inhibitors, phytates, oligosaccharides, glycinin, and β-conglycinin, which reduce nutrient digestion, particularly in young or monogastric animals. Therefore, suitable processing methods are needed to improve SBM quality. Microbial fermentation is an effective approach because it can reduce ANFs, produce organic acids and enzymes, and promote the breakdown of complex proteins into smaller peptides, thereby improving digestibility and nutrient utilization in animals [1]. In addition, fermentation supports current interest in the sustainable use of biological resources by adding value to locally available agricultural materials [2-4].

Lactic acid bacteria (LAB) are attractive starter cultures for fermentation because they utilize carbohydrates in plant-based materials as energy sources and convert them into organic acids, particularly lactic acid. This acidification lowers the pH of the fermentation system and creates unfavorable conditions for spoilage and pathogenic microorganisms. In soybean meal fermentation, LAB may contribute to controlled acidification, fermentation stability, and improvement of feed quality. Previous studies have reported that fermented soybean meal can show increased lactic acid production, lower pH, improved protein degradation, increased small peptides and free amino acids, and reduced oligosaccharides or other antinutritional substances [5-7].

Traditional fermented vegetables are widely consumed, readily available locally, inexpensive, and rich in naturally occurring acid-producing microorganisms. Vegetable fermentation generally relies on microorganisms naturally present on vegetable surfaces and plant tissues. Brining and oxygen-restricted conditions favor salt- and acid-tolerant microorganisms, particularly lactic acid

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bacteria, which often become dominant during fermentation [8-11]. Therefore, traditional fermented vegetables provide a suitable natural habitat for isolating LAB that are adapted to plant-based fermentation environments and may have good acid-producing potential for application in soybean meal fermentation.

During soybean meal fermentation, LAB can use available carbohydrates and added sugar as substrates for lactic acid production, resulting in rapid acidification of the fermentation matrix. The resulting acidic environment may enhance fermentation stability and contribute to the partial degradation of soybean proteins, including glycinin and β-conglycinin, into smaller peptides and free amino acids. These biochemical changes are important because they are associated with improved digestibility and reduced effects of some antinutritional components in fermented soybean meal [7-10]. Therefore, this study aimed to isolate LAB from traditional fermented vegetables, screen the isolates for acid-producing ability, identify the selected isolate, and evaluate its potential for improving acid production during the solid-state fermentation of soybean meal.

MATERIALS AND METHODS

Sample collection

Traditional fermented vegetable samples were randomly collected from 2 local markets in Chonburi Province, Thailand. The samples were aseptically collected in sterile containers and transported to the laboratory under refrigerated conditions. All samples were processed within 24 h after collection for the isolation of lactic acid bacteria.

Culture media

De Man, Rogosa and Sharpe (MRS) agar and MRS broth were used for the isolation, purification, activation, and propagation of lactic acid bacteria [11]. MRS agar supplemented with $CaCO_3$ was used for the preliminary screening of acid-producing colonies (clear zones indicate acid dissolution of $CaCO_3$). [12]. Sterile saline solution was used for serial dilution of the samples.

Isolation and screening of lactic acid bacteria

Traditional fermented vegetable samples were serially diluted from 10^{-1} to 10^{-6} using sterile saline solution. Appropriate dilutions were transferred onto solid MRS agar supplemented with $CaCO_3$ and then incubated at 35°C for 48 h. After incubation, colonies with clear zones around them colonies were selected and purified by transferring them to MRS agar using the streak plate method. The purified cultures were then tested for lactic acid bacteria by Gram stain and catalase testing [13].

Acid production efficiency test in MRS broth

Each strain of LAB bacteria was grown in MRS broth and incubated at 30°C for 24 h to use as the starting cell inoculum. The initial cell density of each isolate was adjusted to the same 1.0 McFarland standard, then transferred into MRS broth and incubated at 30°C. The pH was monitored at 0, 6, 12, 24, 36, and 48 h to assess the acidogenic capacity of each isolate. The isolate showing the greatest reduction in pH was considered to have the highest acid-producing ability and was selected as a representative strain for subsequent soybean meal fermentation experiments.

Solid-state fermentation of soybean meal

1. Starter Culture Preparation: The selected LAB isolate was cultured in MRS broth and incubated at 30°C for 24 h to activate the culture prior to fermentation. Then the culture was then adjusted to the 1.0 McFarland standard using sterile distilled water to be used as a starter culture for the solid fermentation of soybean meal.

2. Preparation of Soybean Meal Fermentation: The experiment consisted of two sample groups: a LAB inoculum group and a control group without inoculum. The fermentation formula consisted of 50% SBM, 10% sugar, and 1% lactic acid bacteria for the LAB inoculum group. DI water was then added to reach 100% (volume by weight of the total substrate). The soybean meal and sugar were thoroughly mixed, and a separate liquid phase was prepared. For the LAB inoculum sample, the bacteria were mixed with water, and the liquid phase was gradually added to the dry mixture, mixing until homogeneous. The prepared samples were then placed into fermentation containers, sealed, and labeled with the sample group, experimental group number, and fermentation start time. Fermentation was carried out at room temperature. Each sample group had three experimental sets. Samples were collected separately at 0, 7, 24, 31, 48, 56, 72, 79, 96, 103, 120, 127, 144, 151, and 168 h to determine the total acid content.

Total titratable acidity analysis

The total titratable acid (TTA) was determined by titration with 0.1 N NaOH and is expressed as percentage of lactic acid (% lactic acid; %LA). The fermented soybean meal sample was mixed with distilled water in a 1:10 ratio. Then, the mixture was homogenized and filtered to obtain the filtrate. An aliquot of 10 mL filtrate was titrated with standardized 0.1 N NaOH using phenolphthalein as an indicator until a pale pink endpoint persisted for approximately 30 s. A blank titration was performed using distilled water under the same procedure. The net volume of NaOH used was calculated as follows:

$$V_{net} = V_{sample} - V_{blank}$$

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Total acidity was calculated and expressed as % lactic acid for solid samples using the following equation:

$$\%LA = \frac{V_{net} \times N \times 90.08 \times V_{total} \times 100}{1000 \times V_{aliquot} \times W}$$

where:

V_{net}= net volume used for titration.
V_{sample}= volume used for the sample.
V_{blank}= volume used for the blank.
N= normality of NaOH.
V_{total}= final volume after sample dilution.
$V_{aliquot}$= volume of filtrate used for titration.
W= sample weight.

The results were expressed as % Lactic Acid (%LA, w/w) for comparison of acid production between the LAB-inoculated samples and the non-inoculated control samples.

Molecular identification of the selected isolate

1. Extraction of Bacterial DNA: Genomic DNA of selected isolates was extracted using a bacterial genomic DNA extraction kit according to the manufacturer's protocol.

2. PCR Amplification of 16S rDNA: The primers and amplification program required for PCR amplification of bacterial 16S rDNA referred to reference [14].

3. Sequencing of Amplified Products: After purification, the 16S rDNA amplification product was sequenced with universal 16S rDNA primers by Shanghai Sangon Biotech Co., Ltd and the obtained sequence was compared with reference sequences available in the NCBI GenBank database using BLAST analysis.

4. Phylogenetic analysis was performed based on the 16S rRNA gene sequence using MEGA12.1.2 software. A phylogenetic tree was constructed to determine the taxonomic relationship between the selected isolate and reference strains.

5. Statistical analysis was performed using SPSS software. All experiments were conducted in triplicate, and data are expressed as mean ± standard deviation (SD, n=3). Differences in pH values among strains were analyzed using one-way ANOVA followed by Games-Howell post hoc test. The effects of treatment, fermentation time, and their interaction on total lactic acid content were analyzed using two-way ANOVA. Statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Description of samples

Two types of traditional fermented vegetables, pickled mustard greens and spider weed, were used as sources for bacterial isolation. Visual observation showed that the samples ranged from light to dark green and consisted of whole stems and leaves immersed in cloudy brine. The samples also had a characteristic sour odor associated with natural fermentation (Figure 1).

Figure 1 Traditional fermented vegetable samples used for bacterial isolation: (A) pickled mustard greens and (B) spider weed.

Isolation and screening of lactic acid bacteria

After isolating bacteria on MRS medium supplemented with $CaCO_3$, acid-producing colonies can be identified by the presence of a clear ring around the colony. This ring is formed when organic acids produced by lactic acid bacteria dissolve $CaCO_3$ in the medium, creating an observable ring around the colony. Based on the presence of clear zones, a total of 9 preliminary isolates were obtained. Six bacterial strains, namely CLT-33, CLT-35, CLT-51, CLT-52, CLT-53, and CLT-L01, were selected after Gram staining and microscopic examination, as well as catalase reaction testing. These strains were selected based on their Gram-positive, catalase-negative, and non-spore-forming characteristics, consistent with the features of lactic acid bacteria. Subsequently, the six strains were tested for their acid-producing ability in MRS broth. The results showed that strain CLT-L01 had a high acid-producing rate. This is an important factor affecting the duration of the fermentation process and serves as a key indicator for evaluating the fermentation ability or robustness of the strain, as shown in Table 1. From Table 1, CLT-L01 showed the fastest acid production rate, with the lowest pH decreasing to 3.21 ± 0.02 after 24 h of incubation. This was followed by CLT-35, which had a pH of 3.46 ± 0.01, and CLT-51, CLT-33, CLT-53 and CLT-52 which had a lowest pH of 3.94 ± 0.03, 4.15 ± 0.02, 4.53 ± 0.02 and 4.85 ± 0.01 after 24 h of incubation, Therefore, based on the acid production rate, CLT-L01 was selected as the strain with rapid acid production for use in subsequent experiments.

Morphological, colony and physiological and biochemical characteristics

1. Cell Morphology: The microscopic observations of strain CLT-L01 revealed that it consisted of short, rod-shaped or nearly spherical cells arranged singly or

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in short chains. These cells were non-motile, non-spore-forming, and measured approximately 0.5-0.8 μm × 1.0-1.2 μm, as shown in Figure 2A. These morphological features are consistent with the general description of members of the genus *Lactobacillus*, particularly *L. plantarum*, which are generally rod-shaped and non-spore-forming [15,16]. Morphological testing combined with Gram staining and the catalase test are the primary screening criteria in the microbiology of fermented foods, being particularly important in identifying gram-positive and catalase-negative lactic acid bacteria [17].

2. Colony Characteristics: The colonies of strain CLT-L01 grown on MRS agar are opaque, spherical, and homogeneous, with a center of approximately 1.2-1.5 mm, slightly convex, smooth surface and have a slight viscosity as shown in Figure 2B. Previous reports indicate that lactic acid bacteria isolated from common fermentation media produce small, smooth, and convex colonies on MRS agar due to acid metabolism and adaptation to a carbohydrate-rich environment [15,17].

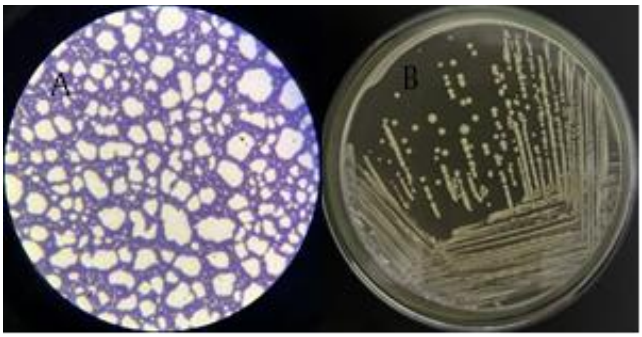

Figure 2 The microscopic appearance of cells (A) and colonies of strain CLT-L01 grown on MRS agar (B).

Table 1 The lowest pH values after culturing 24 hours in MRS broth by six strains of lactic acid bacteria.

Strain	CLT-33	CLT-L01	CLT-35	CLT-51	CLT-52	CLT-53
pH value	4.15 ± 0.01^{d}	3.22 ± 0.02^{a}	3.46 ± 0.01^{b}	3.92 ± 0.03^{c}	4.86 ± 0.01^{f}	4.54 ± 0.02^{e}

* Values are mean ± SD (n = 3), Different superscript letters indicate significant differences among strains (Games-Howell post hoc test, $p < 0.05$).

Solid-state fermentation testing of soybean meal using L. plantarum CLT-L01

The results of the soybean meal test showed that the percentage of total lactic acid (%LA) tended to increase in both the samples inoculated with CLT-L01 and the uninoculated control samples. However, the samples inoculated with CLT-L01 showed a significantly higher effective increase in total lactic acid compared to the control samples ($p < 0.05$), as shown in Figure 3. From figure 3 shows that the total lactic acid inoculated with CLT-L01 increased continuously, rising to 1.16 ± 0.02%, 3.50 ± 0.01%, 6.00 ± 0.21%, and 8.49 ± 0.15% after 7, 24, 48, and 72 h of fermentation, respectively. It then increased to 10.20 ± 0.25% after 96 hours of fermentation and peaked at 11.13 ± 0.06% at 127 hours of fermentation. It then slightly decreased towards the end, remaining at 10.20 ± 0.15%, 8.85 ± 0.30%, and 8.40 ± 0.15% at 144, 151, and 168 h, respectively. While the uninoculated samples showed a gradual increase. The total lactic acid increased to 1.32±0.03% after 48 h of fermentation and peaked at only 2.43±0.12% after 151 h. This demonstrates that the addition of CLT-L01 inoculum promotes lactic acid production during SBM fermentation more efficiently than natural fermentation as seen in the uninoculated group. This is consistent with previous research reporting similar findings on soybean meal fermentation using *Lactobacillus* spp. inoculum, which increases lactic acid production and improves SBM fermentation quality [5,6]. Higher total lactic acid content after fermentation is a desirable characteristic for animal feed products, which can help improve growth performance and balance of beneficial gut microorganisms, comparable to probiotic supplementation [18]. It was found that there was a slight decrease in acid content towards the end of fermentation, which may be related to lower nutrient levels and accumulation of acidic final products, as well as reduced lactic acid metabolism during prolonged fermentation. This is consistent with previous reports concerning the growth of lactic acid bacteria, which indicate that nutrient deficiencies and the accumulation of lactic acid or higher production products can inhibit bacterial growth and limit bacterial metabolic activity [19].

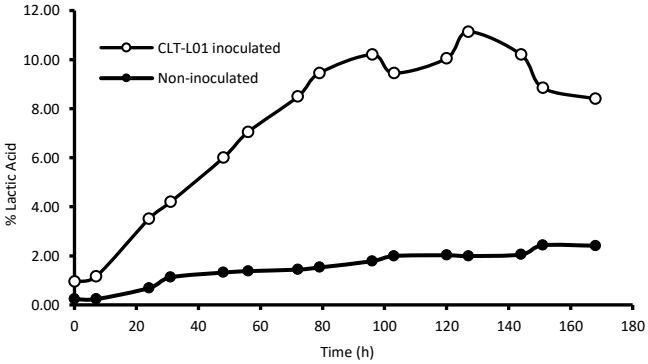

Figure 3 Changes in total lactic acid content in fermented SBM inoculated with CLT-L01 compared with the non-inoculated control during 168 h of fermentation.

Soybean meal served as the solid substrate, and added sugar provided an available carbon source

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for CLT-L01. Under sealed solid-state fermentation, CLT-L01 produced lactic acid from available carbohydrates, acidifying the soybean meal matrix and improving fermentation stability. Thus, the higher lactic acid content in the inoculated treatment reflected the acid-producing ability of CLT-L01 and its compatibility with the sugar-supplemented soybean meal system [5, 6, 19].

However, the total lactic acid content of samples inoculated with CLT-L01 remained higher than that of controls throughout the fermentation period. These findings indicate that CLT-L01 has potential as a starter culture for controlled SBM fermentation and can improve acid production efficiency during fermentation.

Sequence, homology comparison and phylogenetic analysis

To determine the taxonomic position of strain CLT-L01, the 16S rRNA gene was amplified using universal bacterial primers, resulting in a PCR of approximately 1.5 kilobases [14]. The purified amplicons were then subjected to sequencing. The obtained sequences were compared with reference sequences in the GenBank database using BLAST analysis [20]. The results of sequence analysis showed that strain CLT-L01 had more than 99% sequence similarity to the reference strain *L. plantarum*. In addition, phylogenetic analysis based on 16S rRNA sequences showed that strain CLT-L01 belongs to the phylogenetic group of *L. plantarum* and has branching that is consistent with the reference strain. As shown in Figure 4, in the taxonomy of bacteria 16S rRNA sequence similarity has long been used as a key criterion for taxonomic classification. The approximately 97% similarity can be used as a reference for species-level relationships [21,17]. Therefore, the sequence similarity and phylogenetic grouping found in this study can indicate that strain CLT-L01 is closely related to *L. plantarum.* This result is consistent with previous reports that fermented fruits and vegetables are a significant source of the lactic acid bacteria [7-8], particularly *L. plantarum,* a bacterium that is highly adaptable to different fermentation environments and substrates, supporting its potential as a starter culture for plant-based fermentation systems [22-23]. The CLT-L01 strain was then deposited in the culture archive with the access number TISTR P088 to prepare for future development and application.

This study's findings contribute to the development of knowledge and industrial applications by utilizing readily available bio-resources. In this study, low-cost and locally available traditional fermented vegetables were used to isolate potential lactic acid bacteria starter cultures. The selected strain, CLT-L01 (TISTR P088), effectively promoted lactic acid production and acid formation during soybean meal fermentation, suggesting a practical and cost-effective strategy for improving plant-based animal feed formulations. However, further studies on optimizing fermentation conditions, starter culture stability, and product efficacy are needed to support future industrial-scale applications.

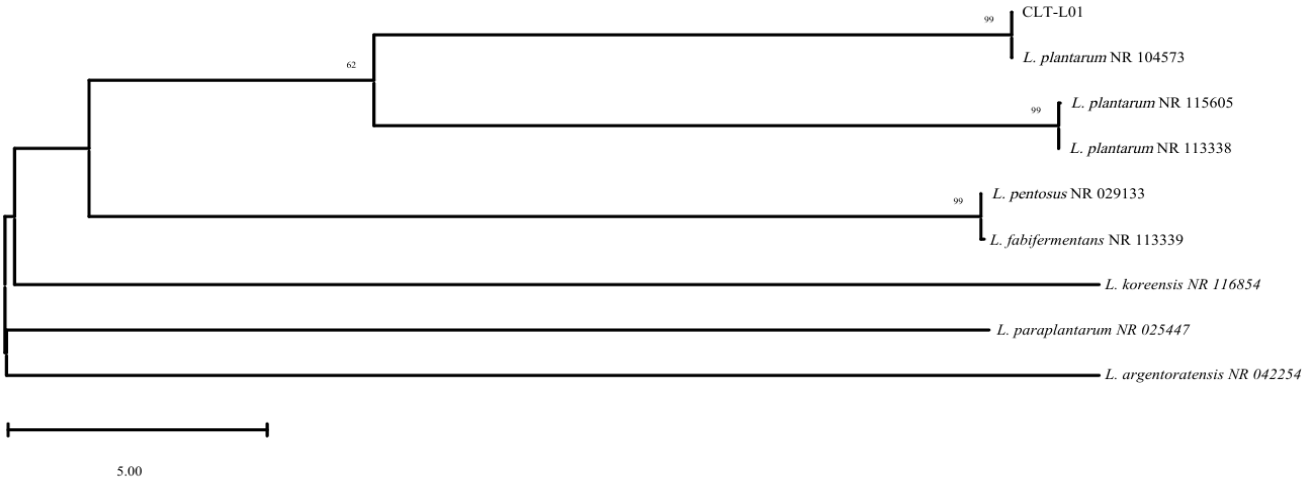

Figure 4 Phylogenetic chart of CLT-L01 isolated from traditional fermented vegetables based on 16S rDNA sequence.

CONCLUSIONS

The selected inoculum CLT-L01 exhibited high 16S rDNA sequence similarity to the reference strain *L. plantarum* and demonstrated efficient acid production capabilities during solid-state fermentation of soybean meal. The lactic acid percentage increased by more than 10% from 96 h onwards, peaking at 11.13 ± 0.06% at 127 hours. These results indicate that CLT-L01 promotes acid production and improves fermentation efficiency compared to natural fermentation. Therefore, CLT-L01 (deposited as TISTR P088) is considered a standout choice as a starter culture for controlling solid-state fermentation of soybean meal.

DECLARATION OF AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

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The authors used Gemini to improve the legibility and clarity of the manuscript during its preparation. The tool was used solely for language editing. All experimental design, data analysis, interpretation and conclusions were carried out by the authors. The authors reviewed and edited the manuscript after using this tool and assume full responsibility for the content of this publication.

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