

Isolation of Bile Salt Hydrolase and Uricase Producing *Lactobacillus brevis* SF121 from Pak Sian Dong (Fermented Spider Plant) for using as Probiotics

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Abstract

The interesting application of bile salt hydrolase enzyme is reduction of cholesterol in serum and amelioration lipid profile. While uricase enzyme can be applied to convert insoluble uric acid to be soluble form and excrete from the body. Probiotics are living organisms with generally know that they can provide beneficial effects to their host. Several reports show that probiotic bacteria with bile salt hydrolase and uricase can improve hypercholesterolemia and hyperuricemia patient. The novel isolate of *Lactobacillus* from Pak Sian Dong in this study is identified as *L. brevis* SF121 and probably use as probiotic bacteria in the future. However, this isolate still need further experiments to investigate and improve properties of probiotics. Moreover, this finding suggests that Pak Sian Dong or fermented spider plant can be designated as a good source for probiotic screening and also defines as health-promoting diet.

Keywords: *Lactobacillus*, bile salt hydrolase, uricase, spider plant

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INTRODUCTION

Lactobacillus is gram positive bacteria in order Lactobacillales and commonly are defined as lactic acid producing bacteria based on their major by-product. This bacteria normally can be found in the environment, dairy product, animal gastrointestinal tract and fermented food.

Several studies have been reported that fermented food can be appropriated as the sources of lactic acid bacteria and also probiotics¹⁻¹⁰. Probiotic bacteria with good properties are usually isolated from fermented vegetable, such as kimchi¹¹ and sauerkraut^{12,13}.

Pak Sian Dong is a kind of fermented vegetable in Thailand, which made from spider plant. Spider plants are affiliated with Cleomaceae family and designated the scientific name as *Cleome gynandra* L. These plants usually are observed worldwide, including Thailand. Fermentation can degrade toxic substance, called hydrocyanic which is defined as a neurotoxin, in spider plant then this kind of food will be safe to consume¹⁴.

Probiotics are assigned as living organism such as bacteria, mold and yeast, which provide advantages to their living host. The first screening criteria for probiotic bacteria are colonizing properties, which are able to attach the surface of the host and can survive in a gastrointestinal tract environment. The other criteria are beneficial properties that promote the health status of the host¹⁵⁻¹⁷.

Deconjugation of bile acid is occurred by catalytic enzyme calls bile salt hydrolase. Various bacteria have ability to produce this enzyme, especially lactic acid bacteria and Enterobacteria. The interesting application for bile salt hydrolase is cholesterol lowering. There are many reports that used bile salt hydrolase producing bacteria for decreasing of cholesterol level in blood of animal models and human volunteers¹⁸⁻²⁰.

Gout is a disease which links to serum uric acid. Resulting of solubility of uric acid in blood is low, high level of this substance can cause precipitation of uric acid crystal leading to inflammation of joints. Therapeutic of this disease is probably aiming at; first, reduction of uric acid production by using allopurinol drug for xanthine oxidase activity inhibition; second,

using probenecid to inhibit tubular reabsorption of uric acid and; third, using pegloticase for acting as uricase enzyme^{21,22}. Uricase or urate oxidase enzyme has ability to transform insoluble uric acid to allantoin which is soluble substance and can be excreted by the kidney. Therefore, catalytic for uric acid activity, uricase is interested to be used for hyperuricemia and gout. Variety genus of bacteria have been screened and isolated with uricase activity, such as *Bacillus*^{23, 24}, *Pseudomonas*²⁵ and *Lactobacillus*²⁶.

The objective of this study was the determination of bile salt hydrolase activity, uricase activity and colonizing properties in *Lactobacillus brevis* which was isolated from Pak Sian Dong in Pathum Thani, Thailand.

MATERIALS AND METHODS

Strain isolation and cultivation conditions

Lactobacillus brevis SF121 was isolated from Pak Sian Dong. Pak Sian Dong were purchased from local area in Pathum Thani province, a gram of each sample was mixed with 9 ml of NaCl, 0.9% (w/v) or normal saline solution (NSS). The mixture was cultured on MRS agar which contained CaCO₃ 0.5% (w/v) and the plates were incubated for 48 h at 37°C incubator. Clear zone from CaCO₃ degradation was observed around the colony and the colony was picked to determine for lactic acid bacteria characteristics. The characteristics were included gram reaction with positive results and catalase test with negative results. Blood hemolytic zone of the isolate was also observed after 37°C incubated at least 72 h. All bacterial isolates were named which including *L. brevis* SF121 and stored at -80°C in glycerol until used.

Bile salt hydrolase activity and uricase activity

For determination of bile salt hydrolase activity of the isolates, MRS medium with 0.5% (w/v) of glycodeoxycholic acid (GDCA) (SigmaAldrich, USA) or taurodeoxycholic acid (TDCA) (Sigma-Aldrich, USA) were prepared as described by Yasiri *et al.*²⁷. The isolates were streaked on GDCA- or TDCA-MRS agar plate and then placed at 37°C for 48 - 72 h. After incubation, colony with halozone was observed and recorded for ability to produce bile salt hydrolase.

To determine uricase activity, plate assay with modification was performed^{23, 25}. The isolate

was streaked on MRS agar containing 0.5% (w/v) uric acid (Sigma-Aldrich, USA) and clear zone were observed after incubation for 48 - 72 h at 37°C.

Strain identification

API50CHL (Biomerieux, France) was used to identify the strain. In addition, the 16s rRNA sequence analysis also was used to confirm species of the strain. The bacterial genomic DNA of the isolate was extracted with GF-1 bacterial DNA extraction kit (Vivantis, Malaysia) and amplification of 16s rRNA by PCR was performed. The forward universal primer F8 (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse universal primer R1492 (5'-GGTTACCTGTTACGACTT-3') were used for amplification. After PCR amplification and agarose gel electrophoresis, Novel Juice (GeneDireX, USA) was mixed with the product and then observed the result on 0.8% (w/v) agarose (Invitrogen, USA). The PCR products were sequenced and the sequencing results were compared with a database (NCBI).

Gastric fluid and bile salt tolerance

To determine the tolerance of gastric fluid and bile salt, the procedure with slightly modified from Mota *et al.* was followed²⁸. Synthetic gastric fluid was prepared as follows; weighed NaCl for 2 g and pepsin for 3.2 g and then mixed in 1000 ml of water. Added concentrated hydrochloric acid to solution to make the pH 2.5 and finally the solution was sterilized by filtration with 0.22 µm pore size membrane. To determine gastric fluid tolerance, bacterial suspension was prepared by washing and adjusting the overnight culture of *L. brevis* SF121 with NSS to McFarland standard no. 0.5. A hundred microliter of the *L. brevis* SF121 cell suspension was applied to 900 µl of synthetic gastric fluid and incubated for 3 h at 37°C. NSS was used instead of synthetic gastric fluid as a control group. The bacterial survival was evaluated and calculated on MRS agar by drop plate technique. For bile salt tolerance test, liquid MRS with 0.3% (w/v) of bile salt was prepared and inoculated with 1% inoculum of *L. brevis* SF121 overnight culture. The numbers of bacteria were determined with the plate count technique at 0 h and 24 h of incubation. The percentage of survival after 24 h in bile containing condition was evaluated to assess bile salt tolerance.

Hydrophobicity

Hydrophobicity at cell surface of *L. brevis*

SF121 was determined as following. Briefly, the overnight culture of *L. brevis* SF121 was washed twice with NSS and centrifuged to harvest the cells. The pellet of bacteria was adjusted to O.D. 0.4 - 0.5 at 600 nm (A0) with NSS. The bacterial suspension from A0 was pipet to test tube for 1.2 ml and mixed with 0.2 ml of xylene. After mixing, O.D. 600 nm measurement of a lower aqueous phase was performed. Calculation of hydrophobicity percentage by using $(1-A1/A0) \times 100$ formula²⁸.

Antibiotic susceptibility

In this study, the MIC of antibiotics, including, ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalidixic acid, penicillin, tetracycline and vancomycin were determined for antibiotic susceptibility following CLSI (Clinical and laboratory standards institute) guideline. In brief, contained antibiotic MRS broth was transferred to a microplate with a serial two-fold diluted from 512 µg/ml to 1 µg/ml. The overnight culture of *L. brevis* SF121 was diluted with NSS to prepare the suspension equal turbidity to 0.5 McFarland. The prepared *L. brevis* SF121 suspension was diluted hundred-fold with MRS and transferred to a filled microplate. The MIC was observed and interpreted after overnight incubation at 37°C.

Antimicrobial activity

The well diffusion assay was used to assess antimicrobial activity of *L. brevis* SF121. The twenty milliliters of Mueller-Hinton agar plates were prepared and swabbed with the indicator organisms. The indicator organisms in this study, including gram positive bacteria (*Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecium*) gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica* Typhimurium, *Shigella sonnei*, *Vibrio cholerae*) and yeast (*Candida albicans*). All organisms were cultivated in Mueller-Hinton broth at 37°C for overnight before spreading on agar plates. A well on swabbed plate that made from cork borer no.3 was filled with 100 µl of *L. brevis* SF121 overnight culture supernatant, which was prior adjusted the pH to 7.0 with 1 N NaOH and filtered by 0.22 µm pore size membrane for sterilization. The plates were measured for the inhibition zone after overnight incubation at 37°C.

RESULTS

Lactobacillus isolation and characterization

Gram positive rod shaped bacteria with catalase negative and no hemolytic activity was selected from MRS agar. Bile salt hydrolase activity was shown as precipitated halozone around the colony of *L. brevis* SF121 on GDCA-MRS agar plate (Fig. 1) but no activity observed on TDCA-MRS. The isolate also exhibited ability to convert insoluble uric acid in MRS agar plate to soluble form which showed as the clear zone around colony in the Fig. 2.

Lactobacillus identification

The selected isolate showed 96.6% identity with *Lactobacillus brevis* by API 50 CHL. The identification was confirmed by 16s rRNA sequencing, which indicated coherent result with 99% identical to *L. brevis* in NCBI database. From isolation and identification, this isolate was designated as *Lactobacillus brevis* SF121.

Determination of probiotic properties

From general probiotic property characterization, gastric fluid tolerance of the isolate in this study was 67.07 % and bile salt tolerance was 89.62%. The *L. brevis* SF121 presented 21.44% hydrophobicity

For antibiotic susceptibility test, the isolate demonstrated sensitivity to only erythromycin and

presented resistance to remaining antibiotics in this study (Table 1). Furthermore, all test organisms in the experiment, including gram positive bacteria, gram negative bacteria and yeast, could not be inhibited the growth by *L. brevis* SF121.

DISCUSSION

From screening and isolation result, *Lactobacillus* species had been isolated from Pak Sian Dong. Previous study showed that the various genus of lactic acid bacteria can be found in Pak Sian Dong, including *Lactobacillus* sp.²⁹, thus the one report reveals potential of lactobacilli from this source have capability to use as probiotic²⁷. There was no hemolytic activity presented on blood agar plate which could be confirmed safety of the isolate for further used in animal and human¹⁷.

Determination of colonizing properties revealed that the isolate can resist simultaneous gastrointestinal environment. Both gastric fluid tolerance and bile salt tolerance are key important properties for survival of probiotic bacteria that appropriate for oral route use³⁰. The properties confirm that this bacteria can survive and provide beneficial effects to the host at gastrointestinal tract region. For hydrophobicity, the isolate

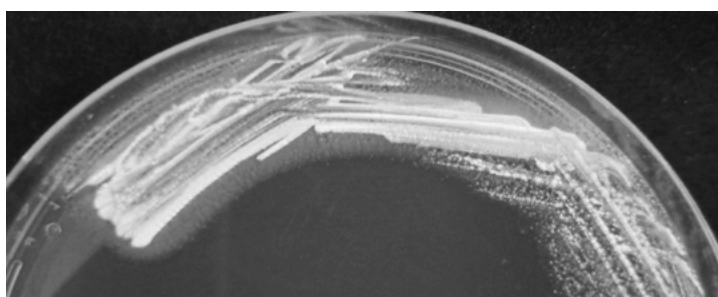


Fig. 1. The *L. brevis* SF121 on GDCA-MRS medium. The activity of bile salt hydrolase showed with halozone surrounding colonies.

Table 1. Antibiotic susceptibility profiles of *L. brevis* SF121 isolate

	Antibiotics (µg/ml)							
	Ampi	Chlo	Cipr	Eryt	Nali	Peni	Tetr	Vanc
<i>L. brevis</i> SF121	32 (Resist)	8 (Resist)	64 (Resist)	0.5 (Sens)	>256 (Resist)	32 (Resist)	16 (Resist)	>256 (Resist)

The antibiotics in this study consisted of ampicillin (Ampi), chloramphenicol (Chlo), ciprofloxacin (Cipr), erythromycin (Eryt), nalidixic acid (Nali), penicillin (Peni), tetracycline (Tetr) and vancomycin (Vanc).

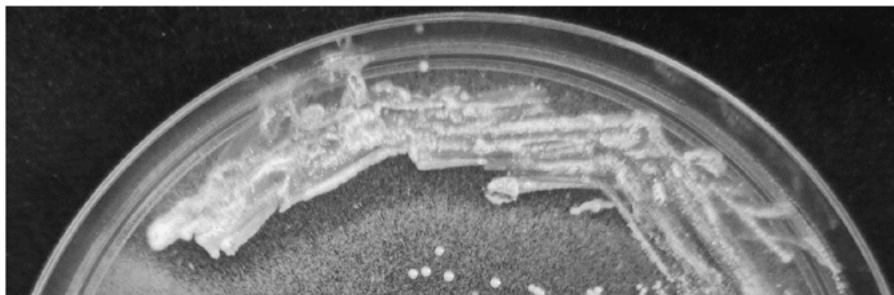


Fig. 2. Uricase activity of *L. brevis* SF121 presented by clear zone on MRS medium containing uric acid.

showed a little percentage, however the bacteria probably use other mechanisms to attach the epithelium cells such as aggregation property³¹.

The novel isolate has been identified as *L. brevis*, which commonly be found in milk, plants and fermented products. This bacteria can be applied to use in industrial and food technology and normally defined as GRAS (Generally Recognized As Safe)³². Some strain of *L. brevis* presented probiotic properties, e.g., antioxidant properties³³, anti-carries activity³⁴, immunomodulating activity³⁵ and synergistic with prebiotic for antimicrobial activity³⁶.

From the result of bile salt hydrolase, the isolate showed the activity only on MRS agar containing GDCA. This finding conformed to previous study that lactobacilli from Pak Sian Dong have an ability to hydrolyze GDCA but not TDCA²⁷. According to bile salt hydrolase activity, this isolate can probably be alternatively used for cholesterol lowering application. Several reports published that lactobacilli, such as *L. plantarum*³⁷⁻³⁹, *L. gasseri*⁴⁰, *L. johnsonii*^{41,42} and *L. brevis*⁴³, could be proved for bile salt hydrolase production. In 2013, *L. plantarum* with bile salt hydrolase was fed in high-cholesterol diet-induced rat could improve lipid profile in serum and liver of the rat⁴⁴. Another report revealed that *lactobacilli* with strong expression of bile salt hydrolase have the capability to ameliorate hypercholesterolemia in mouse model²⁰. In accordance with previous evidences, the novel strain, *L. brevis* SF121, might be able to reduce cholesterol by bile salt hydrolase activity.

Because of uricase enzyme is related to hyperuricemia therapy, this isolate with uricase activity is possibly appropriated to apply in

this therapeutic approach. In human, uricase cannot be produced because of the loss of this enzymatic activity by genetic mutation during Miocene epoch⁴⁵. Therefore, the compensation of this enzyme with intestinal microbes might be an important point. The normal route of administration of uricase (Pegloticase) is intravenous but there is a report that oral uricase administration in animal showed elimination of uric acid^{46,47}. Moreover, alteration of *Lactobacillus* genus show relation with hyperuricemia in animal model⁴⁸ so the adjustment of lactobacilli population probably a helpful for treatment.

CONCLUSION

L. brevis SF121, which isolated from fermented spider plant or Pak Sian Dong in this study, found ability to present bile salt hydrolase and uricase activity. This isolate is probably able to use for reducing of cholesterol and uric acid in person who is defined as hypercholesterolemia and hyperuricemia. This isolate exhibited resistance to gastrointestinal condition and survived in proper time for using as oral probiotic bacteria. Due to possibility to find the bacteria with bile salt hydrolase and uricase activity in Pak Sian Dong, this kind of fermented food would be established as a health beneficial food and also a good source for probiotic bacteria screening. However, there are several properties of probiotic needs to be conducted in future study for probiotic authentication of the *L. brevis* SF121 isolate.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in this manuscript.

ETHICS STATEMENT

This article does not contain any studies with human or animal experiments by any of the authors.

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