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Contributed Paper

β-glucosidase Producing *Bacillus* Isolated from *Thua-nao*, an Indigenous Fermented Soybean Food in Thailand

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ABSTRACT

The characteristics of commercial dried flatten *Thua-nao* collected from Northern of Thailand, namely oven dried (LPO) and sun dried (LPS) samples from Lamphun, sun dried sample from Chiang Mai (CMS) and sun dried sample from Chiang Rai (CRS),were investigated. The variations of chemical and physical characteristics were found among these *Thua-nao* products. Seventeen isolates of spore-forming bacteria were obtained from such products. Among these, four isolates (LPO-2, LPO-4, LPS-2 and CMS-1) were identified as *Bacillus subtilis* group by API 50CHB kit. For cellulolytic enzyme production, CMS-1 exhibited the highest clear zone on tryptic soy agar supplemented with carboxymethyl cellulose ($P<0.05$). Contrastingly, LPO-2 had the highest β-glucosidase activity with the lowest clear zone of cellulolytic enzyme activity ($P<0.05$). From 16S rRNA gene sequencing result, LPO-2 could be identified as *Bacillus* sp. and closely related to *B. subtilis* group.

Keywords: *Bacillus*, β-glucosidase, *Thua-nao*, fermented soybean

1. INTRODUCTION

Thua-nao is a Thai traditional fermented soybean, which is generally consumed in Northern of Thailand. The fermentation of *Thua-nao* is similar to other fermented soybean products described in several countries, i.e. natto (Japanese fermented soybean), cheonggukjang (Korean fermented soybean) and kinema (Indian fermented soybean) [1]. Due to the formation of ammonia during soybean fermentation,

the ultimate pHs ranged from 7.1 to 8.9 [2-4]. After fermentation, the resulting product has unique flavour and taste [4]. Traditionally, the manufacturing process of *Thua-nao* consists of soaking, boiling and fermenting of soybeans. Soybeans is soaked in tap water overnight and boiled for 4 h. After boiling, the water is drained off and cooked soybeans are packed into bamboo basket covered with leaves. The fermentation of cooked soybeans is usually at room temperature within 3 days [2, 4, 5]. The achievement of traditional fermentation process depends on natural microorganisms, existing in the raw materials [4-5]. Among these microorganisms, *Bacillus* genus is frequently isolated from *Thua-nao*such as *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. polymyxa*, and *B.coagulans* [4-7]. *B. subtilis*, a Gram-positive, endospore-forming bacterium, has usually been found as the predominant microorganism in *Thua-nao* [8]. This strain has been paid more attention as an alternative fermentative microorganism for soybean fermentation according to its ability to increase the nutritional value of the fermented products, such as increasing of essential amino acids [1, 3], enhancing of antioxidant properties [3, 9] and increasing of isoflavone aglycone [6, 7, 10]. The latter is of importance in functional food manufacturer due to the increase in elderly population.

During fermentation of soybeans, microbial β-glucosidase enzyme can break sugar moiety and increase isoflavone glucoside hydrolysis, resulting in higher concentration of isoflavone aglycones [10-12]. The differences in bioactivity and metabolic activity of dietary soybeans isoflavones depend on chemical forms. The structure of isoflavone is a limiting factor for absorption in the gastrointestinal tract. The aglycone forms are more readily absorbed and more bioavailable than isoflavone glucosides [11-13]. Recently, the β-glucosidase activity produced from *B. subtilis* and *B. pumilus* in fermented soybeans has been reported [6, 7, 14]. Also, β-glucosidase producing *B. subtilis* contributed to an increase in isoflavone content during black soybean pulp fermentation [11]. From the points of view, the increase in isoflavone aglycones content could be increased during soybean fermentation, particularly *Thua-nao*, with β-glucosidase producing *Bacillus* sp. inoculation. However, the information regarding the β-glucosidase producing *Bacillus* sp. isolated from *Thua-nao* has not been reported. Therefore, the objectives of this study were to isolate β-glucosidase producing *Bacillus* sp. from *Thua-nao*and to identify the strain showing highest β-glucosidase activity by polymerase chain reaction (PCR). Also, the chemical and physical characteristics of *Thua-nao* samples were reported.

2. MATERIALS AND METHODS

2.1 Sample Collection

Fresh *Thua-nao* or "*Thua-nao kab*" samples were collected within 1-3 day(s) of manufacturing from Lamphun (LP), Chiang Mai (CM) and Chiang Rai (CR) provinces during November to December, 2013. All samples were certificated from a Thai Community Product Standard (TCPS) [15]. Samples were separately kept in the plastic bags with tightly sealed and stored at room temperature (30-32°C) until analysis (within 1 week).

2.2 Physicochemical Properties Analysis

Moisture and salt contents were determined according to the AOAC methods [16]. Water activity (a_w) was determined using a water activity meter (AquaLab series 4, AquaLab, USA) and pH was determined according to the method

of Benjakul *et al.* [17] using a pH meter (UltraBASIC, Denver Instrument, NY). Colour was determined using a colourimeter (ColorFlex, HunterLab, USA) and reported as L*, a* and b*.

2.3 Isolation and Characterisation of Spore-forming Bacteria from *Thua-nao*

Ten grams of sample were mixed with 0.85 g/100 mL NaCl (w/v) with vigorous shaking and placed in a water bath at 80°C for 20 min [5]. Then, aliquot of suspension (100 µL) was spread on tryptic soy agar (TSA) (Merck, Darmstadt, Germany). After incubation at 37°C for 24 h, distinct morphology colonies were selected and isolated by streaking on TSA. Then, all isolates were maintained on TSA slant and kept at 4°C for further analyses.

Cell morphology and Gram's staining were examined by light microscope (CX41, Olympus, Japan). Phenotypic characterisation, including oxygen requirement, catalase test, Voges-Proskauer reaction and hydrolysis of starch, were carried out as described by Norris *et al.* [18]. Carbohydrate utilisation was performed by using an API50CHB kit (BioMérieux, France) according to the manufacturer's instruction. *Bacillus subtilis* TISTR001 obtained from Thailand Institute of Scientific and Technological Research (TISTR), Thailand was used as a reference strain.

2.4 Screening of β-glucosidase Producing *Bacillus*

2.4.1 Growth profile analysis

The production of secondary metabolites depends on the growth phase of microorganisms. Thus, the highest activity of cellulolytic enzyme was found at the late log phase of *B. subtilis* cultured in TSB [5, 19-20].Growth profiles of all isolates were monitored every 2 h for 24 h by measuring the optical density at 600 nm using a spectrophotometer (GeneQuant1300, Canada). To correlate the optical density with cell growth, plate count method on TSA was also employed. Briefly, a single colony from TSA plate of each isolate was cultivated in TSB with shaking incubation at 150 rpm and 37°C. At each time interval, optical density of each bacterium was measured by using TSB as blank. Plate count was performed on TSA with appropriate dilution in 0.85 g/100 mL NaCl. After incubation at 37°C for 24 h, colonies were counted and expressed as log colony forming units (log CFU/mL).

2.4.2 Crude enzyme preparation

Crude enzyme was prepared as described by Kim *et al.* [19] and Wongputtisin *et al.* [20]. One loop of bacterial cell on TSA slant was transferred to 50 mL of TSB supplemented with 0.1 g/100 mL carboxymethyl cellulose (CMC) (Merck, Darmstadt, Germany). After shaking incubation at 37°C for 24 h (150 rpm), one millilitre was transferred to 50 mL of TSB supplemented with 0.1 g/100 mL CMC. At late log phase of each isolate, cells were removed by centrifugation at 10,000 ×*g* for 20 min at 4°C. The supernatant was collected and filtered through a 0.22 µm polyethersulfone filter (Whatman, UK). The filtrate was used as crude enzyme solution.

2.4.3 Screening of cellulolytic enzyme activity

Cellulolytic enzyme activity of eachisolate was determined as described by Wongputtisin *et al.* [20] with a slight modification. Thirty microlitres of crude enzyme were placed in the wells (0.5 cm, ∅) punched on TSA supplemented with 0.1 g/100 mL CMC. After incubation at 37°C for 48 h, plates were stained with 0.2 g/100 mL Congo red

for 15 min and then washing with 1 mol/L NaCl for 30 min. The cellulolytic enzyme activity was measured diameter of clear zone by a Vernier caliper (NAZA, China) and expressed as clear zone diameter (mm).

2.4.4 β-glucosidase Activity Assay

β-glucosidase activity was performed according to the method of Cho *et al.* [7] with a slight modification. The β-glucosidase activity was measured by using *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma, USA) as a substrate. Crude enzyme (500 µL) was added to 250 µL of 5 mmol/L *p*-NPG (pH 7.0). After incubation at 37°C for 60 min, the enzymatic reaction was terminated by adding 1 mL of 0.2 mol/L glycine-NaOH (pH 10.5) and the absorbance was immediately measured with spectrophotometer (Gene Quant1300, Canada) at 405 nm. The blank solution was composed of 500 µL of TSB, 250 µL of substrate solution and 1 mL of 0.2 mol/L glycine-NaOH (pH 10.5). The *p*-nitrophenol (*p*-NP) released by the action of the enzyme was determined by referring to a calibration curve prepared from *p*-NP in concentrations that varied from 0 to 300 µmol/L. One unit (U) of β-glucosidase activity was defined as the amount of enzyme that liberated 1 µmole *p*-NP/mL/min.

2.5 Identification of Selected β-glucosidase Producing *Bacillus* by 16S rRNA Gene Sequencing and Phylogenetic Analysis

16S rRNA gene of strain which showed the highest β-glucosidase activity was amplified by polymerase chain reaction (PCR). Briefly, bacteria cells grown on TSA plate were collected and DNA was extracted according to the protocols of Kawasaki *et al.* [21], Yamada *et al.* [22] and Katsura *et al.* [23]. The amplification reaction was performed by using the universal bacteria primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) annealed at positions 8-27 and 1513R (5′-TACGGTTACCTTGTTACGACTT-3′) annealed at position 1492-1513 (*E. coli* numbering according to Brosius *et al.* [24]) with a volume of 100 mL of a reaction mixture contained 15-20 ng of template DNA, 2.0 mmole of each primer, 2.5 units of *Taq* polymerase, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphate and 10 mL of 10x*Taq* buffer (pH 8.8) containing $(NH_4)_2SO_4$ which composed of 750 mmol/L Tris-HCl, 200 mmol/L $(NH_4)_2SO_4$ and 0.1 mL/100 mL Tween 20. Amplification was carried out in a thermocycler (BioRad Laboratory) with the following cycling program: initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min and a final amplification step at 72°C for 30 min. The PCR product was analysed by 0.8 g/100 g agarose gel electrophoresis and purified using GenepHlow™ Gel/PCR kit (Geneaid, Taiwan). The double-stranded DNA was sequenced with an ABI Prism (Applied Biosystems, USA) by the use of the following four primers; 27F and 518F (5′-CCAGCA GCCGCGGTAATACG-3′) annealed at positions 518-537 as a forward primers and 800R (5′-TACCAGGGTATCTAATCC-3′) annealed at positions 783-800 and 1513R as a reverse primers. The 16S rRNA gene sequence was assembled using BioEdit program (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html). The assembled sequence was submitted to the GenBank database. The sequence was aligned along with selected sequences form the GenBank/EMBL/DDBJ databases by using the CLUSTAL_X program (version 1.81) [25]. Gaps and ambiguous bases were eliminated. A phylogenetic tree was constructed using the neighbor-joining method [26] and

the MEGA (version 6.0) program [27]. Confidence values for individual branches in the phylogenetic tree were determined using the bootstrap analysis of Felsenstein [28] based on 1,000 samplings.

2.5 Statistical Analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests ($P<0.05$). Statistical analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, Ill., USA).

3. RESULTS AND DISCUSSION

3.1 Physicochemical Properties of Commercial *Thua-nao* Samples

The physicochemical properties of commercial *Thua-nao* samples are shown in Table 1. Moisture content of LPO, LPS, CMS and CRS were 9.25, 12.89, 12.76, and 11.51 g/100 g, respectively. As a result, themoisture content of all samples wass atisfied by the specification of TCPS [15]. The lowest moisture content can be found in oven-dried sample (LPO), compared with other sun-dried samples ($P<0.05$). According to the other reports, the moisture content of dried flatten *Thua-nao* was varied, for instance, 11.88 g/100 g [2], 7.60 g/100 g [29], and 7.30-12.02 g/100 g [30]. The difference in water activity (a_w) of samples was also observed. LPO had the lowest a_w, compared with other samples ($P<0.05$). Generally, dried food products have $a_w$less than 0.60; meanwhile, intermediate moisture foods (IMF) have $a_w$ranging between 0.60-0.85 [31]. From the result, LPS, CMS and CRS can be classified as IMF but LPO can be classified as a dried food. The pH values of LPO, LPS, CMS and CRS were 5.68, 5.70, 5.85 and 5.83, respectively ($P<0.05$) (Table 1). This indicated that all samples were low-acid food. Chukeatirote and Thakang [2] reported that pH of dried *Thua-nao* was 5.9. In general, the a_w and pH values could be used for shelf-life determination of food products [32]. Salt (as sodium chloride) content of all samples was found in the ranges of 0.09 - 2.16 g/100 g. LPS had the highest salt content followed by LPO, CMS, and CRS, respectively ($P<0.05$). Generally, sodium chloride is an important adjunct in fermented food due to its many useful functions, particularly to lower a_w and to control microbial growth [33]. The fermented *Thua-nao* is generally added with 3 g/100 g salt before drying. From the results, the characteristics of dried flatten *Thua-nao* samples were depended on drying methods and amount of salt added. Differences in pH and salt content of samples might be affected by the different manufacturing processes, including preparation, boiling, and fermentation (temperature, relative humidity and starter cultures) as well as drying process.

The highest L* value was observed in LPO ($P<0.05$) (Table 1). L* value indicated the lightness of samples. From the observation, LPO exhibited light brownish (Figure 1) and other samples showed darker brownish colour. This might be resulted from the differences in drying conditions as well as in the chemical compositions between samples. For a* and b* values, CMS showed the lowest a* and b* values, followed by CRS, LPO and LPS, respectively ($P<0.05$). Different colour of fermented soybean products can occur due to the variations in variety of soybean used, boiling time of cooked soybean, fermentation conditions and period of soybean incubation [4]. Some factors (pH, a_w, temperature and substrate) can promote both enzymatic and non-enzymatic browning reactions [34-35].Generally, soybean is rich in phenolic compounds, which can be oxidized by

polyphenol oxidase in presence of oxygen to develop reddish-brown *o*-quinones [34]. The enzymatic browning would occur prior to heat treatment whereas the amine-carbonyl reaction or Maillard reaction can be taken place at all stages of manufacturing process, particularly with high temperature. Therefore, Maillard reaction can be regarded as the crucial coloured reaction affecting the degree of browning in *Thua-nao* products.

Table 1. Characteristics of commercial *Thua-nao* samples.

Parameters	LPO	LPS	CMS	CRS	TCPS [15]
Moisture content	9.25±0.07^{c}	12.89±0.34^{a}	12.76±0.10^{a}	11.51±0.36^{b}	≤13
Water activity	0.57±0.02^{d}	0.70±0.01^{c}	0.75±0.00^{b}	0.77±0.00^{a}	-
pH	5.68±0.02^{b}	5.70±0.01^{b}	5.85±0.05^{a}	5.83±0.02^{a}	-
Salt content	1.99±0.05^{b}	2.16±0.07^{a}	0.14±0.01^{c}	0.09±0.00^{c}	-
Colour					
L*	44.70±0.05^{a}	36.56±0.16^{b}	21.42±0.79^{d}	27.93±0.87^{c}	
a*	9.57±0.28^{b}	10.68±0.21^{a}	5.31±0.28^{d}	8.32±0.29^{c}	
b*	22.71±0.30^{b}	24.63±0.41^{a}	7.54±0.25^{d}	11.48±0.61^{c}	

Means ± SD from triplicate determinations with different superscript letters in the same row indicate significant differences (P<0.05).

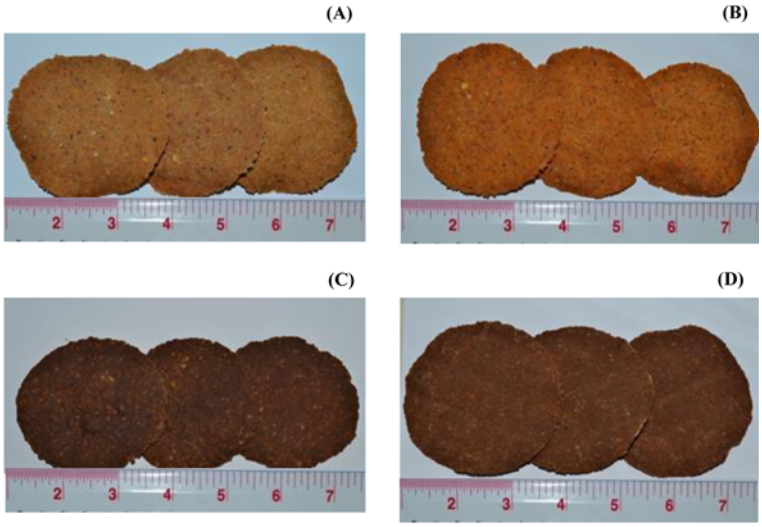

Figure 1. Commercial *Thua-nao* samples; LPO (A), LPS (B), CMS (C) and CRS (D).

3.2 Isolation and Characterisation of Spore-Forming Bacteria from *Thua-nao*

Seventeen spore-forming bacteria were isolated from *Thua-nao* samples. Among them, eight isolates were selected according to their distinctive morphologies on TSA plate as LPO-2, LPO-4, LPS-1, LPS-2, LPS-3, CMS-1, CRS-2 and CRS-5. After cultivation on TSA at 37°C for 24 h, the morphology of colonies was varied from white to cream colour with flat to umbonate appearance. The colony of isolates were wavy, opaque and non-glistening, except LPS-2 showed smooth to wavy edge and glistening. The colonies

of LPO-4 and LPS-3 looked like slimy. All isolates were Gram-positive, rod shape, endospore-forming, and aerobic growth. Under microscopy, the size of all of all isolates were varied (0.5-1.25 × 2.5-4.0 μm), while CRS-5 displayed the longest cell size (1.25 × 4.0 μm). All isolates gave positive results for catalase test, Voges-Proskauer test and hydrolysis of starch, indicating the production of catalase enzyme, acetylmethylcarbinol (acetoin) and exoenzymes, including α-amylase and oligo-1, 6-glucosidase (starch hydrolysis), respectively. Comparing the results obtained from *B. subtilis*TISTR001 (reference strain), all isolates can be characterised as *B. subtilis* group according to the simplified key for the tentative identification of typical strains of *Bacillus* species [18]. In general, *B. subtilis* can produce several enzymes and other useful biological compounds, leading to a reason for its superiority in the soybean fermentation [5-8, 36].

Carbohydrate utilisation of all isolates was examined with an API50CHB kit in comparison to the reference as shown in Table 2. The obtained results showed that the similarity between LPO-2, LPO-4, LPS-2, CMS-1 and *B. subtilis*TISTR001 were 99.9%, 99.9%, 99%, 93.7% and 97.3%, respectively. From the results, these four strains were selected for further screening of β-glucosidase activity.

Table 2. Differential carbohydrate utilisation of strains LPO-2, LPO-4, LPS-2, CMS-1 and *B. subtilis* TISTR001 using API50CHB test.

Carbohydrate utilisation	*B. subtilis* TISTR001	LPO-2	LPO-4	LPS-2	CMS-1
D-Xylose	-	+	+	+	+
D-Mannose	+	-	+	+	+
L-Sorbose	-	-	+	-	-
Inositol	+	+	+	+	-
D-Sorbitol	+	+	+	+	-
N-acetyl glucosamine	+	-	-	-	-
Arbutin	-	+	-	-	-
Salicin	-	+	-	+	-
D-Cellobiose	-	+	+	+	-
D-Maltose	+	+	+	+	-
D-Lactose	-	-	+	+	-
D-Melibiose	-	-	-	+	-
Inulin	+	+	-	-	+
D-Raffinose	-	-	-	+	+
Starch	v	+	v	+	+
Glycogen	-	+	+	+	+
Gentiobiose	-	-	+	+	-
D-Turanose	+	-	-	-	-

+, positive reaction; -, negative reaction; v, variable.

3.3 Screening of β-Glucosidase Producing *Bacillus*

Figure 2 depicts the growth curve plotting between cell density and total count. It was found that the late log phase of LPO-2, LPO-4, LPS-2 and CMS-1 were 8, 8, 8 and 6 h, respectively. Thus, samples at late log phase were collected and used for determination of cellulolytic enzyme and β-glucosidase activities.

The cellulolytic enzyme activity of four strains was indicated by clear zone diameter (Figure 3). Clear zone diameter of isolates LPO-2, LPO-4, LPS-2 and CMS-1 were 8.86, 22.03, 10.65 and 29.67 mm, respectively ($P<0.05$) as shown in Figure 4A. CMS-1 had the highest cellulolytic enzyme activity, while LPO-2 exhibited the lowest cellulolytic enzyme activity ($P<0.05$). In general, cellulolytic enzyme is a multi-enzyme, including endoglucanase (CMCase) that hydrolyse the exposed cellulose chains of cellulose polymer, exoglucanase (cellobiohydrolase) that act to release cellobiose from the reducing and non-reducing ends, β-glucosidase (cellobiase) that help to cleave the cellobiose and short-chain cello-oligosaccharide into glucose, filter paperase (FPase), avicelase, and xylanase [19-20, 37]. Although the mechanism of cellulose degradation by aerobic bacteria is similar to that of aerobic fungi, it is clear that anaerobic bacteria operate on a different system [37-39]. A large number of microorganisms can produce cellulase as cell bound or extracellular[40].In addition, *Bacillus* species can secrete cellulase, including strains of *B. subtilis* [38], *B. cereus* [39], *B. licheniformis* [40] and alkaliphilic *Bacillus* [41]. However, the diameter of clear zone may not accurately reflect the real cellulase activity, particularly β-glucosidase [38-41].

The activity of β-glucosidase from four strains (LPO-2, LPO-4, LPS-2 and CMS-1) and reference (*B. subtilis* TISTR001) is depicted in Figure 4B. The activity of β-glucosidase of reference, LPO-2, LPO-4, LPS-2 and CMS-1 were 0.32, 0.32, 0.28, 0.31, and 0.30 U/mL/min, respectively ($P<0.05$). Among four strains tested, LPO-2 exhibited the highest β-glucosidase activity ($P<0.05$), even though its cellulolytic enzyme activity was the lowest (Figure 4A). The results indicated that the β-glucosidase activity of LPO-2 was found to be a major component in cellulolytic enzyme. The β-glucosidases from microorganisms can be generally intracellular, extracellular or membrane bound. Intracellular β-glucosidases are synthesized after exhaustion of carbon source in the medium [42]. Rani *et al.* [43] suggested that the extracellular β-glucosidase can be led to easily separation. As a result, the β-glucosidase activity in crude extract was likely resulted from both extracellular and membrane bound enzymes. The β-glucosidase activity regarding to the cleavage of the β-glycosyl bond in soybean isoflavone glucosides to form aglycone has been paid more attention [44]. Generally, human can either absorb aglycones at intestine or metabolise by intestinal microflora to equol and *O*-desmethylangolensin[45]. Aglycone isomers are able to bind to estrogen receptor sites and hence mimic the function of estradiol in human body [46]. Recently, the β-glucosidase activity from *Bacillus* spp. isolated from soil has been reported. Kim *et al.* [19] reported that β-glucosidase activity of *B. subtilis* strain SL9-9 was 0.2 U/mL. The β-glucosidase activity of *B. pulmilus* EB3 was 0.038 U/mL [47]. Also, the increase in isoflavone aglycones during fermentation of choenggukjung inoculated with *B. pumilis* HY1 and *B. subtilis* CS90 isolated from Korean soybean sauce (kanjang) has been reported [6-7]. From the results, strain LPO-2 with the highest β-glucosidase activity was chosen for identification by 16S rRNA gene sequencing and phylogenetic tree analysis.

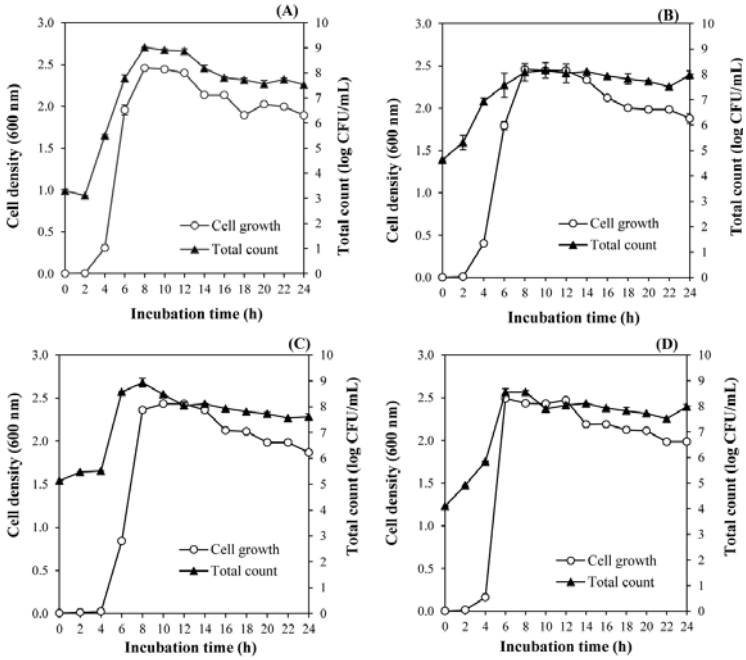

Figure 2. Growth profile of isolated strains as followed: LPO-2 (A), LPO-4 (B), LPS-2 (C) and CMS-1 (D).

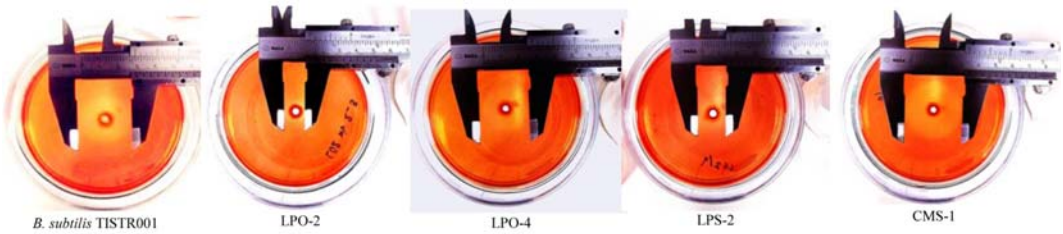

Figure3. Cellulase activity measurements on TSA supplement with 0.1 g/100 mL (w/v) CMC agar plates developed by Congo red staining.

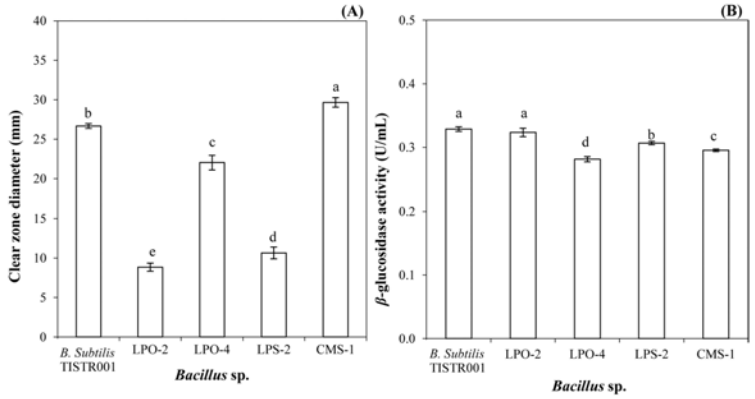

Figure 4. Size of clear zone formed by the cellulolytic enzyme activity (A) and β-glucosidase activity (B) produced by some *Bacillus* sp. isolates. One unit of β-glucosidase activity was defined as 1 µmole*p*-NP/min/mL.

3.4 Identification of Selected β-glucosidase Producing *Bacillus* by 16S rRNA Gene Sequencing and Phylogenetic Tree Analysis

The sequence of 16S rRNA gene (1415 bp) of strain LPO-2 was obtained from sequencing analysis and submitted to the GenBank database with the accession number of KU321253. Then the sequence was aligned along with available 16S rRNA sequences in the GenBank/EMBL/DDBJ databases. Phylogenetic tree constructed by using the neighbor-joining method is shown in Figure 5. Phylogenetic analysis using the 16S rRNA gene sequence indicated that strain LPO-2 belonged to the genus *Bacillus* and closely related to *B. tequilensis* KCTC 13622^{T} (accession number AYTO01000043) in the cluster of *B. subtilis*. Moreover, LPO-2 shared 99.72% similarity with *B. tequilensis* KCTC 13622^{T} (accession number AYTO01000043) and *B. subtilis* subsp. *inaquosorum* KCTC 13429^{T} (accession number AMXN01000021), and 99.65%, 99.58%, 99.58% and 99.50% with *B. subtilis* subsp. *subtilis* NCIB 3610^{T} (accession number ABQL01000001), *B. subtilits* subsp. *spizizenii* NRRL B-23049^{T} (accession number CP002905), *Brevibacterium halotolerans* DSM 8802^{T} (accession number AM747812) and *B. mojavensis* RO-H-1^{T} (accession number JH600280), respectively, with 1-5 bp different. As taxonomic group of *B. subtilis* subsp. *subtilis*, *B. subtilits* subsp. *spizizenii*, *B. subtilits* subsp. *inaquosorum*, *B. mojavensis*, *B. tequelensis* and *Brevibacterium halotolerans*, could not distinguishable by 16S rRNA gene sequence, thus biochemical properties of this taxonomic group have to be concerned [48]. Distinguishing phenotypic characteristics of strain LPO-2 and related *Bacillus* species are presented in Table 3. Whereas the comparative analysis of the 16S rRNA gene sequences from strain LPO-2 and *B. tequilensis*, *B. subtilis* and *B. mojavensis* would suggest no phylogenetic distinction, evidence from phenotypic characterisation study justify recognition of strain LPO-2 as representing of *B. subtilis* group. Thus, this strain was identified as *Bacillus* sp. within *B. subtilis* group.

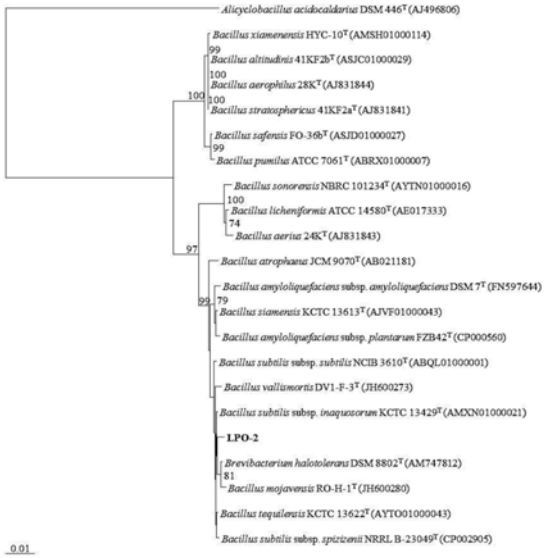

Figure 5. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain LPO-2, some *Bacillus* species and related taxa. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at the branch points Bar, 0.02 substitution per nucleotide position.

Table 3. Differential characteristics of strain LPO-2 and related *Bacillus* species.

Characteristics	Strains			
	LPO-2	*B. tequelensis**	*B. subtilis**	*B. mojavensis**
Decomposition of:				
Tryptophan	-	+	-	-
Starch	+	-	+	+
Decomposition under anaerobic conditions of:		+	-	-
Arginine	-	+	-	-
Lysine	-	+	-	-
Ornithine	-	+	-	-
Production of indole	-			
Acid production from:		+	-	+
Galactose	-	+	+	-
β-Gentiobiose	-	+	+	-
Glycogen	+	+	+	-
Melibiose	-	+	+	-
Methyl-α-D-glucoside	+	+	-	+
Rhamnose	-	+	+	-
D-Turanose	-			

*****Data from Gatson *et al.* [49].
Data were obtained in this study unless otherwise indicated. +, positive reaction; -, negative reaction.

4. CONCLUSION

Commercial *Thua-nao* samples were varied in chemical compositions and physicochemical characteristics, including moisture content, a_w, salt content, pH, and colour. The variation was probably owing to the differences in raw material composition, preparation, fermentation, drying methods and storage conditions. Eight spore-forming bacteria isolated from *Thua-nao* samples could be classified as *Bacillus* spp. by morphological, physiological and biochemical characteristics. In addition, the results obtained from API 50CHB test could identify LPO-2, LPO-4, LPS-2 and CMS-1 as *Bacillus* sp. within *B. subtilis* group. Based on the activities of cellulolytic and β-glucosidase enzymes, the selected strain, LPO-2, could be identified as *Bacillus* sp. which closely related to *B. subtilis* group. Thus, LPO-2 can be used as an alternative starter culture for *Thua-nao* fermentation with enhancement of isoflavone aglycones.

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