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A taxonomic study of *Bacillus* sp. strain S1-13 isolated from Terasi, an Indonesian shrimp paste

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Abstract

A bacterial strain S1-13 was screened and isolated from Terasi, a traditional fermented seafood product of Indonesia. This isolate, producing an extracellular protease with high activity when culturing on casein agar, was studied in terms of their phenotypic and genotypic properties including morphology, biochemical profiles, and rRNA gene sequence. The isolate S1-13 was a rod-shaped, endospore-forming, Gram-positive bacterium. Further analysis based on the results of its biochemical profiles and 16S rRNA gene sequence, showed that the strain S1-13 belonged to the *B. subtilis / B. amyloliquefaciens* group. Additionally, the bacterial isolate S1-13 was also evaluated for its antibiotic susceptibility and it was found to be susceptible to most antibiotics tested (chloramphenicol, erythromycin, kanamycin, tetracycline and vancomycin), intermediately susceptible to bacitracin, and resistant to ampicillin.

Introduction

Terasi is the traditional fermented seafood product of Indonesia. Generally, Terasi is made from tiny shrimp or often from a mixture of fish and shrimp. The product is popular and widely used as condiments in various local dishes (Surono and Hosono, 1994a). Traditionally, Terasi can be processed in two ways: i) with salt only, and ii) with salt and other ingredients (Yunizal, 1998). In general, the shrimp is washed, drained, and dried. Impurities (i.e., shells and coral) are removed during this step. The semi dried shrimp is then pounded and mixed with salt. The amount of salt used is varied ranging from 2 to 5% of the shrimp weight. The mixture is exposed to sunlight and allowed to ferment under this condition (i.e., at ambient temperature) for 1 – 4 weeks before packing for sale (Yunizal, 1998). In Indonesia, a variety of Terasi products are found resulting from difference in raw materials used, production process, fermentation condition, and geographical location (a source of fermenting microbiota). There are other products related to Terasi which are found in Malaysia (Belachan), Myanmar (Ngapi), the Philippines (Bagoong), and Thailand (Kapi).

To date, liteature dealing with Terasi is limited. Surano and Hosono (1994a, b) reported the microbiological analysis of Terasi. Some Terasi starters exhibiting halophilic property were isolated and studied (Kobayashi *et al*., 2003; Setyorini *et al*., 2006). In the present study, the protease-producing bacteria were screened and isolated from Terasi. The bacterium isolate S1-13 exhibiting strong proteolytic activity was studied and reported.

Materials and Methods

Screening and isolation of protease-producing bacteria

The Terasi sample used in this study was from Cirebon (north coast of West Java). Initially, ten grams of Terasi were transferred to 90 ml of sterile 0.1% peptone and homogenised in a stomacher. The suspension (1 ml) was then serially diluted in 9 ml of sterile peptone and suitable decimal solutions (0.1 ml) were spreaded on casein agar. After incubating at 37°C for 24 h, the colonies exhibiting clear zones were selected and subcultured to obtain the pure cultures.

A single colony of these bacteria was subcultured into a test tube containing 3 ml of nutrient broth. The culture was incubated at 37°C for 24 h. Fifty microlitres of the overnight culture was transferred to a new test tube containing the nutrient broth (3 ml) and the culture was further incubated at 37°C for 24 h. After incubation, the bacterial cells were harvested (14,000 rpm, 15 min, 4°C). The supernatant was collected and used in the protease activity assay.

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For this, ten µl of the supernatant were spotted on a skim milk agar plate (containing 0.5% peptone, 0.3% beef extract, 0.5% skim milk, and 1.5% agar). The inoculated plate was then incubated at 37°C for 24 h. The presence of a clear zone was recorded. The proteolytic activity assay was carried out in triplicates. The value of the relative index of protease activity was calculated from the ratio between the diameter of the clear zone and that of the bacterial colony, and thus used to indicate the proteolytic activity of the bacterial strains.

Morphology and biochemical profiles

The selected bacterium S1-13 was characterised by morphological, and biochemical properties. These features included Gram-staining, presence of spore, oxygen requirement, catalase test, lecithinase test, ability to growth in 5 and 7% NaCl, growth at 50 and 65°C, IMViC test, nitrate reduction, fermentation of glucose, arabinose, xylose and sucrose, and starch hydrolysis, based on the protocol of Slepecky and Hemphill (1992).

Phylogenetic analysis

The 16S rRNA gene sequence of the bacterium S1-13 was retrived from GenBank (an accession no. JX441363) (Benson *et al*., 2013). To investigate taxonomic relationship, the 16S rRNA gene sequence of the bacterium S1-13 was aligned and compared with those of the type strains of the related *Bacillus* species. For this, the bacterial type strains used were *B. subtilis* DSM10 (AJ276351), *B. licheniformis* DSM13 (X68416), *B. amyloliquefaciens* ATCC23350 (X60605), *B. megaterium* IAM13418 (D16273), and *Lactobacillus plantarum* ATCC14917 (AJ621668). The phylogenetic analysis was then inferred from this alignment using the Phylogeny.fr software (Dereeper *et al*., 2010).

Susceptibility tests

Antimicrobial susceptibility was performed as instructed by NCCLS (1997). Antibiotic discs (Oxoid, England) used in this study were vancomycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), ampicillin (30 µg), bacitracin (10 U), and streptomycin (10 µg).

Results and Discussion

Proteolytic activity of Terasi bacteria

In this study, we aimed to investigate the protease-producing bacteria from Terasi, an Indonesian shrimp paste. In total, 26 bacterial isolates were obtained from Cirebon Terasi. Of these, 15 isolates representing 58% were able to produce the protease enzyme when culturing on skim milk agar as indicated by the presence of the clear zone (Table 1). It is interesting to note that the ability to produce the protease of these bacteria varied as observed by the relative index (RI) value ranging from 0.90 to 2.78. This variation is also possible due to the enzyme efficiency (i.e., a suitablility of substrate), and thus will be subject to further study. Most bacterial isolates (8 isolates) exhibited a slightly weak proteolytic activity activity (with the RI value between 1.01 – 1.50). The bacterium strain S1-13 had the highest protease activity showing the RI value of 2.78, and hence was selected to study further in terms of its taxonomy. Besides, the protease enzyme produced by the strain S1-13 is being studied and preliminary reported by Yingchutrakul *et al.* (2011). Terasi starters were studied and consisted of a wide range of bacterial group (Surono and Hosono, 1994a,b). However, previous work dealing with Terasi starters focused on halophiles which were understandable when considering from the fact that Terasi is processed in the presence of high salt concentration (Kobayahsi *et al*., 2003; Setyorini *et al.*, 2006). This present work thus extends the research of Terasi bacterial starter.

Table 1. Protease-producing bacteria isolated from Cirebon Terasi. The data shown are the relative index (RI) value calculated from the ratio between the diameter of the clear zone and that of the bacterial colony

Strains	RI value	Strains	RI value	Strains	RI value
S1-1	1.49	S1-6	1.35	S1-11	1.67
S1-2	2.33	S1-7	2.00	S1-12	1.33
S1-3	2.33	S1-8	1.18	S1-13	2.78
S1-4	1.22	S1-9	1.04	S1-14	1.41
S1-5	2.38	S1-10	1.04	S1-21	0.90

Identification of the bacterium strain S1-13

The bacterium strain S1-13 was morphologically studied and subject to a series of biochemical tests as shown in Table 2. The isolate S1-13 was Gram positive, endospore-forming, rod-shaped bacteria. According to its biochemical profiles (i.e., facultative anaerobic, catalase and nitrate reductase positive reactions, production of acetylmethylcarbinol and acid from glucose), the S1-13 isolate showed similar profile to that of *Bacillus subtilis* and *B. amyloliquefaciens* (Welker and Campbell, 1967; Priest *et al.*, 1987).

To confirm the identity, the 16S rRNA gene sequence of the strain S1-13 (accession no. JX441363) was determined initially using a BLAST search GenBank database (Zhang *et al*., 2000). The 16S rRNA gene sequence of the strain S1-13 showed highest similarity (99.68%) to *B. amyloliquefaciens*

Table 2. Morphological, physiological and biochemical characteristics of the bacterium strain S1-13 isolated from Terasi

Characteristics	S1-13	*B. amyloliquefaciens*	*B. subtilis*
Gram	Positive	Positive	Positive
Shape	Rod	Rod	Rod
Presence of spore	+	+	+
Oxygen requirement	Facultative	Facultative	Facultative
Growth in 5% NaCl	+	+	+
Growth in 7% NaCl	-	+	+
NB 50 °C	-	+	+
NB 65 °C	-	-	-
Catalase	+	+	+
Voges Proskauer	+	+	+
Methyl red	-	-	+
Formation of indole	-	-	-
Starch hydrolysis	+	+	+
Citrate utilization	+	+	+
Nitrate reduction	+	+	+
Egg-yolk lechitinase	+	+	+
Acid from glucose	+	+	+
Gas in glucose	-	-	-
Fermentation of arabinose	+	+	-
Fermentation of glucose	+	+	+
Fermentation of xylose	+	+	+
Fermentation of sucrose	+	+	+

Notes: + = positive; - = negative.

and *B. subtilis*. Phylogenetic tree was then generated to determine the phylogeny of the strain S1-13 and its related *Bacillus* species (Figure 1). According to Figure 1, the strain S1-13 was in the same clade of *B. subtilis* and *B. amyloliquefaciens*. It should be noted, however that, for many years, the genus *Bacillus* is known to be complex, and it is thus difficult to differentiate them at the species level. *B. subtilis* in particular is a good example as several previously identified *Bacillus* species are closely related to *B. subtilis* exhibiting similar phenotypic and genotypic characteristics. It is now widely accepted that *B. subtilis* should be considered as 'sensu lato' consisting of *B. amyloliquefaciens, B. licheniformis, B. mojavensis*, and *B. sonorensis* (Rooney *et al*., 2009). Inability to use phenotypic, biochemical, and 16S rRNA gene for species within the *B. subtilis* species complex raises the problem to many microbiologists and those interested in *Bacillus* taxonomy. Recently, an attempt to use multilocus sequence has been proposed (Perry *et al.*, 2007). For the time being, we thus propose that the isolate S1-13 was identified in *B. subtilis / B. amyloliquefaciens* group.

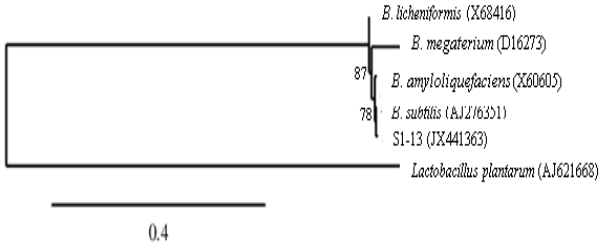

Figure 1. Phylogenetic relationships of the bacterial strains S2-3 and S4-5 with the type strains of other *Bacillus* species. The dendogram was constructed based on the similarity of the 16S rRNA gene sequences (accession numbers are given in parentheses). Bootstrap values greater than 60% were illustrated. The branch length is proportional to the number of nucleotide substitutions per site

Antimicrobial susceptibility

Few studies are dealing with the sensitivity of *Bacillus* species to antibiotics. This is possibly because the majority of *Bacillus* species are not pathogens and, besides, many *Bacillus* species have been used in biotechnological industry (Schallmey *et al*., 2004). The bacterium strain S1-13 showed resistance to ampicillin whereas it showed sensitivity to chloramphenicol, erythromycin, kanamycin, streptomycin, tetracycline and vancomycin (data not shown). This information can be considered as a fundamental phenotypic data related to the strain taxonomy. Besides, based on the data obtained, the use of the strain S1-13 in industrial applications is promising considering from its origin and strong proteolytic activity.

Conclusions

The bacterium S1-13 isolated from Terasi was selected for study because of its high proteolytic activity. In terms of its phenotypic, biochemical, and genetic properties, this isolate was classified as *B. subtilis / B. amyloliquefaciens*. The data obtained especially those of biochemical tests are expected to provide some key characteristics of the S1-13 isolate

which may be useful for future study dealing with the bacterial cultures especially those screened and isolated from similar foods.

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