Food-grade protease producing bacteria isolated from Indonesian soybean tempe gembus and red oncom after prolonged fermentation

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Submission date: 03-Aug-2022 08:17AM (UTC-0400)
Submission ID: 1878413638
File name: 4._2021_05_PROC_SNE_IOP_ICGRC_Sulistyaningtyas_et_al.pdf (367.47K)
Word count: 4475
Character count: 25619

IOP Conference Series: Earth and Environmental Science

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To cite this article: AR Sulistyaningtyas *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **743** 012008

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Abstract. The search of food-grade proteases allowing broad application in food industry and protein modification is on increase worldwide due to pressure on the market of these enzymes as commodity product, both on price reduction and increasing performance. This paper reports nine protease-producing bacterial strains as new sources of food-grade protease from bacteria isolated from two Indonesian fungal fermented foods, soybean Tempe gembus and red oncom. Isolation was conducted on every other day within 5 days of storing (post fermentation) and protease production tests were then carried out on skim milk media agar. Cells of the isolated proteolytic strains were morphologically identified using SEM (Scanning Electron Microscope) and a bacterial phylogenetic tree was constructed using MEGA 7 program based on partial sequences of 16S rRNA genes of these bacteria. Based on taxa analysis and plate-based pathogenicity test, *B. megaterium* IROD3 and, *B. amyloquefaciens* IROD2, *B. tequilensis* ISTD3, and *S. carnosus* IROD4 appeared to be the potential candidates of food-grade proteolytic enzyme producers. This study concluded that prolonged fungal fermentation of soybean Tempe gembus and red oncom allowed growth of known low - nonpathogenic protease producing bacteria.

1. Introduction

Underlining the importance of enzyme, in the concept of Biotechnology set by Food and Agriculture Organization (FAO) in 2017 enzyme is defined as "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" [1]. The use of enzymes in several industries such as agriculture, chemicals, pharmaceuticals and food, has risen speedily due to reduced processing time, low energy input, cost effectiveness, nontoxic and eco-friendly characteristics [2]. In 2014, the industrial enzymes market was globally assessed about $4.2 billion and expected to raise approximately 7 % over the period from 2015 to 2020 for $6.2 billion [3]. Currently, 75% of industrial enzymes are hydrolytic in nature [4].

Hydrolytic enzymes, consisting of protease, lipase, amylase, cellulase, pullanase, etc. are actively produced by various types of bacteria. The search of new sources of bacterial hydrolytic enzymes potential to be used in various industries and applications including bioremediation and degradation and has been intensively conducted worldwide [5-11]. So far, proteolytic enzymes are the greatest vital ones representing global sales of about 60% the total enzyme market in industrial sector [4].

Proteases are a group of enzymes occupying a pivotal position with respect to their applications in both physiological and commercial fields [6]. As degradative enzymes, they catalyze total hydrolysis of proteins. The unique catalytic activities of proteases make them an inexpensive choice for hydrolyzing peptide bonds for industrial uses [5]. Actually, proteases might remain distinguished among hydrolases for their manufacturing uses, which is a collection of enzymes of the furthest application in the food trade, followed by transferases [12].

Proteases as well as other enzymes have been commercially generated from plants, animals, and microbial sources [13]. However, proteases produced by microorganisms have increased attention for their uses in industries and medicine purposes due to the stability, catalytic activity, production and optimization [2]. Microbial enzymes have the enormous benefits of being able to produce in large scale quantities through fermentation techniques [13]. Fermentation, both solid and submerged ones have been widely used for the production of proteases [14,15].

In food industry, proteases have been widely used as food processing aids. Proteases used in the food industry include Alcalase®, Neutrase®, Esperase®, ProtamexTM, and Novozym® FM, which are commercially marketed by Novozymes, Denmark. These bacterial proteases are used for improving the functional, nutritional and flavor properties of proteins. Protease can be applied in the alcohol production to improve yeast growth [16]. For baking purpose, protease used to degrade proteins in flour. In brewing, it is commonly used for removing necessary proteins from malt and barley and obtaining the desired level of nitrogen nutrients. Protease also elaborate in lactose reduction and flavor adjustment in dairy applications [17,18].

Food-grade proteases are proteases useful in food-grade process. Proteolytic enzymes commonly used for baking, food processing and protein modification [4]. As stated by FAO, microbial sources, which may be natural strains or derived from natural strains or variants the processes of selective genetic modification, could be used in enzyme preparations. However, production strains for food-grade protease enzyme should be non-pathogenic and non-toxic [1].

Current progresses in industrial biotechnology caused in the exploitation of new and undiscovered microorganisms and the development of better methods for enzyme production led to increased yields of the enzyme. The primary step of industrial enzyme manufacture process is to isolate a microbial strain that have the potential to produce the enzyme in marketable produces. Extracellular microbial products are screened using a plate assay and the organism's production ability can be detected by hydrolysis zone around the colony [18]. Today, the search for microbial sources of novel proteases in natural diversity is also targeting a hitherto undiscovered wealth of molecular diversity [19].

Indonesian fermented foods, oncom and soybean cakes, have been known are potential sources of bacterial proteases. It has been reported that two bacterial isolates, *Bacillus licheniformis* and *Bacillus pumilus*, which were found in both types of fermented foods as protease producers [7,20]. In the mentioned study, however, it was not clearly stated whether the sample used was fresh or prolonged fermented ones. In fact, there have been no reports about biodiversity of protease producing bacteria, which could be found after prolonged fermentation on these foods. The objective of this study was to report bacterial strains capable of producing protease isolated from red oncom and soybean Tempe gembus after prolonged fermentation (1-5 days).

2. Materials anda Methods

2.1. Materials

This paper summarized isolation and molecular identification of protease producing bacteria from two types of Indonesian fermented foods, soybean Tempe gembus and red oncom. First, Literature study was initiated to summarize required data from publications related with isolation of proteolytic bacteria from Indonesian post-fermented food, soybean Tempe gembus and oncom. Then the summarized data were subjected to further work and analysis including of deposition of DNA

sequence of 16S rRNA gene fragment from each bacterial isolate to Genebank, DNA sequence analysis and alignment, construction of phylogenetic tree based on the deposited 16S rRNA gene fragment sequences using MEGA 7 [21]. software, and morphology analysis of the selected nonpathogenic bacterial cells using scanning electron microscope (SEM).

2.2. Summarization of 16S rRNA PCR products

Visualization of PCR amplification products of 16S rRNA gene fragment from each of 9 bacterial isolate were summarized. This work was done to confirm that single bands sized ~1500 bp matched to theoretical size of amplified 16S rRNA gene sequences could be obtained from each of 9 bacterial genomes isolated in previous work. Data of protease production and 16S rRNA gene fragment sequences of 9 pure proteolytic bacterial were combined.

2.3. Deposition of DNA sequence to Genebank and Construction of Phylogenetic Tree

Each of 9 sequences of 16S rRNA gene fragment from 9 bacterial isolates was submitted to genbank database through Nucleotide Sequence Submission System (NSSS) of DDBJ (DNA Database of Japan) from website https://www.ddbj.nig.ac.jp/submission-e.html following instructions therein. The aim was to obtain sequence accession number for each of these sequences. Name of strains ISTD and IROD were applied referring to the origin of bacterial samples: Indonesian soybean Tempe gembus and red oncom, respectively. and. After submissions were granted, access codes of each sequences were used to construct phylogenetic tree in order to determine the taxonomy status of bacterial isolates. The alignment of 9 bacterial DNA sequences was conducted using ClustalW available in MEGA 7 software [21,22]. The aligned output was then used to construct phylogenetic tree with neighbor-joining algorithm [23].

2.4. Pathogenicity Test and Morphology identification of Bacterial cells

Pathogenicity levels of the obtained strains were qualitatively determined by plate-based pathogenicity test using MacConkey and Blood Agar Media. Further confirmation on pathogenicity of species was obtained from literature. The selected less to non-pathogenic strains were further identified morphologically by subjecting their bacterial cells to SEM observation.

3. Result

Nine strains of bacteria from Indonesian fermented foods capable of producing protease have been isolated and reported to have capacity to produce proteases. Four of these bacteria were isolated from post-fermented soybean Tempe consisting of *Bacillus cereus, B. thuringiensis, B. tequilensis,* and *Pseudomonas stutzeri.* Five others were isolated from post-fermented red oncom encompassing *Bacillus thuringiensis, Bacillus amyloliquefaciens, B. megaterium, Staphylococcus carnosus,* and *S. hominis.* For molecular identification, isolation of 16S rRNA gene using PCR had been conducted. In this study, 9 targeted DNA sequences of these bacterial strains obtained from the amplification of 16S rRNA partial genes using PCR is summarized. Visualization of PCR products from bacterial isolates obtained from the work was combined in Figure 1.

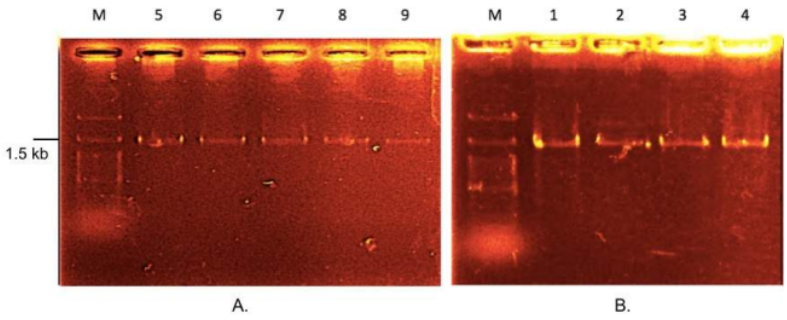


Figure 1. Visualization of PCR products on agarose gel representing amplified DNA of 16S rRNA gene of bacteria isolated from Indonesian soybean Tempe (A) and red oncom (B). DNA bands on electrophoresis gel are labelled as follows M= marker, 1 = strain ISTD1, 2 = ISTD2, 3 = ISTD3, 4 =ISTD4, 5 = IROD1, 6= IROD2, 7 = IROD3, 8 = IROD4, and 9 = IROD5.

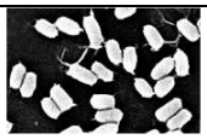
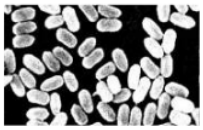
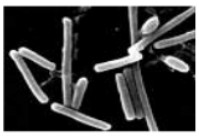
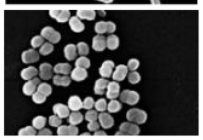
Table 1. Proteolytic bacterial strains isolated from post-fermented Indonesian red oncom and soybean Tempe along with plate-based pathogenicity test results and supporting information required to justify their benefit as sources of food-grade proteases (low-pathogenic strains are green-highlighted).

Strain name (Genbank acc. number)	**Type of post fungal fermented foods**	**Diameter of proteolytic zone (mm)**	**Closest related species (%)**	**BAP test result**	**MacConkey test result**	**Plate-based Pathogenicity Score**
ISTD1 (LC406761)	Soybean Tempe gembus after 1 day	79.0	*B. cereus KAVK5* (98%)	ß	Non-lactose fermenter	High
ISTD-2 (LC406759)	Soybean Tempe gembus after 2 days	64.0	*B. thuringiensis RA2* (98%)	ß	Non-lactose fermenter	High
ISTD-3 (LC406763)	Soybean Tempe gembus after 3 days	56.0	*B. tequilensis VTCC-B-270* (98%)	γ	Lactose fermenter	Low
ISTD-4 (LC406758)	Soybean Tempe gembus after 4 days	79.0	*P. stutzeri E141* (98%)	γ	Non-lactose fermenter	Medium
IROD1 (LC406760)	Red oncom after 1 day	79.0	*B. thuringiensis TERI SID4* (98%)	ß	Non-lactose fermenter	High
IROD-2 (LC406764)	Red oncom after 2 days	29.0	*B. amyloliquefaciens A1142* (98%)	γ	No growth	Low
IROD3 (LC406762)	Red oncom after 3 days	72.0	*B. megaterium OS-66* (98%)	γ	No growth	Low
IROD4 (LC406765)	Red oncom after 4 days	6,0	*S. carnosus JX-1* (99%)	γ	No growth	Low*
IROD5	Red oncom	66.0	*S. hominis K23*	γ	Lactose	Low**

(LC406766) after 5 days (99%) fermenter

* Known as "food-grade" *Staphylococcus* [24]
**In some publications reported (rarely) as opportunistic causing breast abscess, therefore is not included as safe species [25]

Table 2. SEM analysis results on Strain ISTD3, IROD2 and IROD4.

Identified strain	Deposited Genbank Accession No.	SEM observation on bacterial cells	Shape based on SEM observation
Bacillus tequilensis ISTD3	LC406763		Short rod
Bacillus amyloliquefaciens IROD2	LC406764		Short rod
Bacillus megaterium IROD3	(LC406762)		Long rod
Staphylococcus carnosus IROD4	LC406765		Round

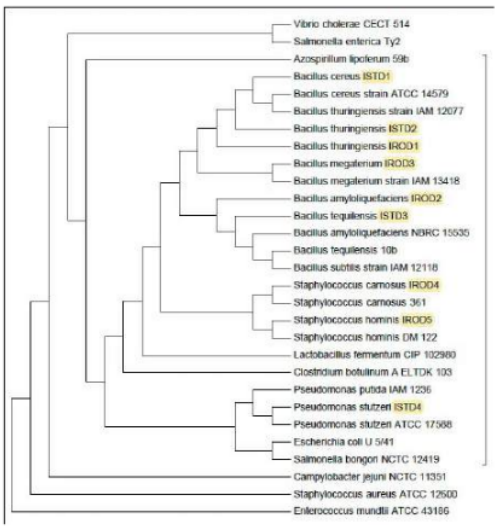


Figure 2. Mega 7 - constructed phylogenetic tree of the 9 obtained proteolytic strains (yellow-highlighted) among other bacterial strains based on 16S rRNA gene sequences retrieved from Genbank database.

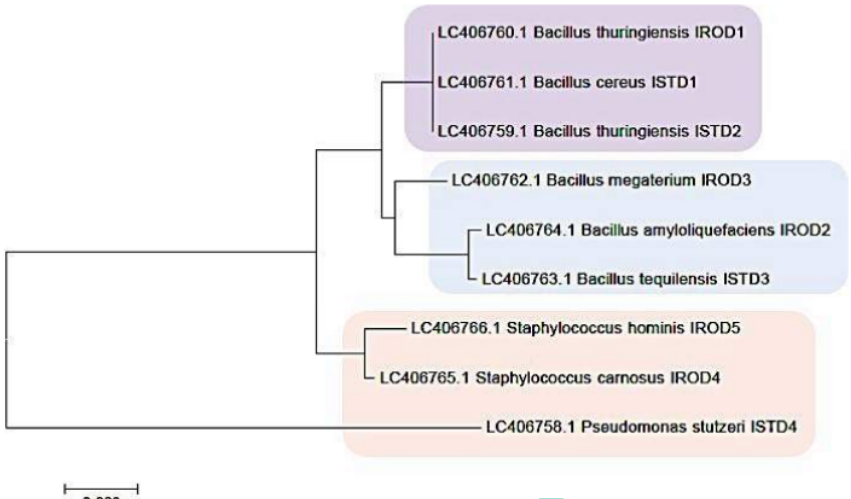


Figure 3. Mega 7 -constructed phylogenetic tree of the 9 isolated proteolytic strains from prolonged fermented soybean Tempe gembus and red oncom samples based on 16S rRNA gene sequences showing closeness between groups of strains isolated in nearly similar days: day 1-2 (purple-highlighted), day 2-3 (blue-highlighted) and day 4-5 (pink-highlighted).

4. Discussion

This paper reports isolation and molecular identification of protease producing bacteria from two types of Indonesian fermented foods, soybean Tempe gembus and red oncom. Four samples of fresh soybean Tempe and 5 samples of fresh red oncom were allowed to undergo prolonged fermentation up to 4-5 days, and bacterial isolation was conducted every day during this prolonged fermentation from each sample. Bacterial isolation was conducted on day 1, 2, 3, 4, and 5 of fermentation initially on nutrient agar media. A single unique colony was selected each day and then purified three times until uniform cells could be observed under optical microscope. As results, 9 pure colonies with distinct shape of each were selected to be tested for their protease production ability.

Based on position of all DNA bands in Figure 1, the presence of amplified partial 16S rRNA gene of the 9 bacteria targeted at 1,5 kb size using universal primers could be confirmed. DNA sequences of PCR products obtained were then deposited in Genbank through DDBJ Japan. The assigned Genbank accession codes of each sequence of the reported 9 pure bacterial isolates are displayed in Table 1. Meanwhile, to measure the ability of pure colonies obtained from all samples to produce protease, cultivation of each colony on skim milk agar (SMA) medium was performed and their proteolytic indexes were measured. The results were also summarized in Table1. Review on species related reports of the 9 strains in terms of benefit or role and case or disease is also displayed in Table 1. Using Genbank accession numbers obtained (listed in Table 1) as references, a phylogenetic tree was constructed using MEGA 7 program for 9 species obtained from both post-fermented soybean Tempe (strains ISTD1-ISTD4) and red oncom (ISTD1-ISTD5). A tree (Figure 1) was resulted as output of MEGA 7 using 16SrRNA sequences in Fasta format as input, which were then proceed using phylogenetic analysis tools.

Based on Table 1, among all obtained protease producing isolates, 4 bacterial strains, ISTD3, IROD2, IROD3, and IROD4 appeared to be the potential candidates of food-grade proteolytic enzyme producers based on characteristics of their related species: *B. tequilensis*, *B. amyloliquefaciens*, *B. megaterium*, and *S. carnosus*. Taxa analysis and further literature studies on pathogenicity supported their low pathogenicity. However, still further molecular investigation on the presence of genes responsible for toxicity of these species is worth done.

Based on results in Table 1, further morphology identification using SEM are subjected to the 4-selected species, and the results could be seen in Table 2. The aim was to confirm their cell

morphology characteristics as part of species identification. as seen from Table 2, SEM results showed that cellular morphology of the observed strains were in-line with their molecular identification results.

Molecular identification on the selected species was conducted referring their 16S rRNA Genbank accession numbers. A phylogenetic tree was constructed using MEGA 7 program for all proteolytic species obtained from both post-fermented soybean Tempe (strains ISTD1-4) and red oncom (ISTD1-5). A tree (Figure 2) was resulted as output of MEGA 7 using 16SrRNA sequences in Fasta format as input, which were then proceed using phylogenetic analysis tools

To understand more about relationship between the 21 bacterial isolates studied, another phylogenetic tree involving only 9 isolated proteolytic strains based on 16S rRNA gene sequences was constructed (Figure 3). As seen on Figure 3, there is relationship closeness between groups of strains isolated in nearly similar days. The tree also reveals that *where B. thuringiensis* appeared as the common (shared) strain found in both types of fermented food samples after prolonged fermentation. Analysis on the resulted tree in Figure 3 showed 3 distinct branches consisting three taxa of each (shaded in purple, blue and pink colors). Interestingly leaves (members) of each branch represent from both types of fermented foods, Tempe and oncom.

The purple group consist of species obtained on day 1-2 of prolonged fermentation both soybean Tempe and red oncom. This group consists of *Bacillus cereus* and *Bacillus thuringiensis* species. *B. cereus* is widely reported as toxigenic [26]. *B. thuringiensis* crystal proteins (*cry*) are well known to be toxic to certain insects but not pathogenic to mammals. Interestingly, the *cry* proteins are selectively toxins to human cancer cells [27].

As seen in Figure 3, the blue group consists of species isolated on day 2-3 of prolonged fermentation of both soybean Tempe and red oncom. In these group, 2 bacterial species refer to low pathogenic bacteria could be found, *B. amyloliquefaciens* and *B. tequilensis*. *B. amyloquefaciens* is widely used as probiotics capable of producing thermophile protease [28-30].

The pink group encompasses species obtained on day 4-5 of prolonged fermentation. Pathogenic species appear in this group, i.e. *Staphylococcus hominis* and *Pseudomonas stutzeri*. *P stutzeri* is known as species that could cause bacteremia, which could lead to death [27]. However, in this group, a less to nonpathogenic species, *Staphylococcus carnosus* could be found. *S. carnosus* is a nonpathogenic species, yet playing important role in meat fermentation [32]

Microbial enzymes utilized in food processing are usually sold as *enzyme preparations* containing not only a targeted enzyme activity but also other metabolites of the production strain, as well as extra materials such as preservatives and stabilizers. The added materials must be food-grade and suit applicable regulatory standards. The production strain safety still is the main consideration when it comes to enzyme safety, particularly the toxigenic effect of the produced-strain. Systematically categorized nonpathogenic, nontoxigenic microbial strains, particularly those pretence histories of safe use in food enzyme manufacture, are rational candidates for producing a safe strain lineage [33].

Based on results of this study, prolonged fermented Indonesian soy bean Tempe and red oncom are great sources of protease producing bacteria, both pathogenic and nonpathogenic ones. Although micro-fungi are the fermentation agent in both protein-rich foods, bacterial strains actively producing protease with high protease index are found to be widespread therein. This study has demonstrated that prolonged fermented soybean Tempe gembus and red oncom are potential source of food-grade protease producing bacteria. It could also be inferred from the results that prolonged fermentation of Indonesian red oncom and soybean Tempe allowed growth of various protease producing strains, where types of bacteria on similar prolonged fermentation day might share close relationship.

5. Conclusions

Prolonged fermented Indonesian soy bean Tempe and red oncom appear to be great sources of proteolytic bacteria. Nine bacterial strains capable of producing protease isolated from red oncom a 24 soybean Tempe gembus after prolonged fermentation (1-5 days) in this study are *Bacillus cereus*, *B. thuringiensis, B. amyloliquefaciens B. tequilensis. B. amyloquefaciens* and *Staphylococcus carnosus*.

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