

Differences in Protease Expression of Mutants *Bacillus licheniformis* F11

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DOI: <http://dx.doi.org/10.13005/bbra/1871>

(Received: 06 October 2015; accepted: 19 November 2015)

The objective of the study was to investigate the differences in protease productions of mutans *Bacillus licheniformis* F11. In this study *B. licheniformis* F11.1 (DchiA; Dpga) and *B. licheniformis* F11.4 (DchiAB; Dpga) was used. Two types (LB and MLB + collagen) of media were used for bacterial growth, protease production and zymogram analysis : Luria Broth (LB) and Modified Luria Broth plus collagen (MLB + collagen) media. The LB media contained tryptone 1%, NaCl 1% dan yeast extract 0.5%. The Modified LB + collagen media contained tryptone 0.5%; NaCl 1%, yeast extract 0.25% and collagen 5%. Two protease producing *B. licheniformis* F11 mutans were screened from Palembang Indonesia, *Bacillus licheniformis* F11.1 (DchiA; Dpga) and *Bacillus licheniformis* F11.4 (Dchi AB; Dpga). Both mutants showed different growth pattern and protease production in media containing collagen. F11.1 displays a higher protease activity than F11.4 in Luria Bertani media with an optimum after 15 hours for F11.1 and 15-25 hours for F11.4. Upon addition of collagen to LB medium F11.4 overmatched F11.1 with respect to collagenolytic activity. The presence of collagen led to a shift in the substrate preference of the two strains towards collagen compared to other protein substrates (gelatin, fibrin and casein). Zymogram analysis on gel embedded substrates suggests a higher molecular variation for strain F11.4. The different genetic mutation of the same species (*Bacillus licheniformis* F11.1 and F11.4) displayed different protease production patterns and substrate specificities especially on collagen.

Key words: Protease, *Bacillus licheniformis* F11.1 and F11.4, *Bacillus licheniformis* F11.1 and F11.4, expresion.

Proteases are involved in numerous cellular operations, such as protein metabolism, post translational modifications, extracellular enzyme secretion, sporulation, and even blood

coagulation^{1,2}. There are non specific proteases, which find applications as washing-detergents as well as in the leather and food industry and for waste processing. Specific proteases are applied as medical agents for treating diseases such as indigestion, arteriosclerosis, stroke, as cosmetic-ingredients but also for molecular biological purposes. Application of specific proteases to marine and plant proteins were shown to be important for the production of a panoply of bioactive molecules such as peptides with antiviral,

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antioxidant and inhibitory effects of the Angiotensin Converting Enzyme³⁻⁵.

Microbial proteolytic enzymes are classified as serine proteases (E.C.3.4.21), sulfhydryl proteases (E.C.3.4.22), metallo proteases (E.C.3.4.24) and acid proteases (E.C.3.4.23). Though several microorganism are known as potent protease producers : *Bacillus subtilis* CN2⁶, *Bacillus* sp.⁷, and *Bacillus subtilis* PE-11⁸. Representatives of the genus *Bacillus* constitute the major bacterial workhorses for commercial enzyme production. Bacilli encode a great number of secreted enzymes, including proteases, amylases, glucanases, etc⁹. Genes encoding secreted enzymes have been cloned and functional analyses as well as genetic regulation was studied in detail as for *Bacillus megaterium*¹⁰⁻¹². The ubiquitously occurring *Bacillus licheniformis* (in many respects a Generally Regarded As Safe or GRAS organism) plays the most prominent role since it produces up to 25 g/l extracellular proteins⁶. The availability of its genome sequence further facilitates use of *B. licheniformis* as a valuable enzyme producer^{13,14}.

In a search for protease producing microorganisms which can be applied for deproteinization of chitin samples derived from shrimp waste, *Bacillus licheniformis* F11 was isolated from shrimp waste in Palembang and characterized as the most promising candidate¹⁵. Amino acid alignments of predicted chitinases revealed 99 to 100% identity to the known *B. licheniformis* enzymes, however, a frameshift mutation was detected in *chiA* of the strain favouring the strain for the above purpose as it almost completely lacks chitinase activity but hypersecreted proteases¹⁵. By a targeted gene deletion approach two mutants of *B. licheniformis* F11 were generated: (1) *B. licheniformis* F11.1 which in addition to the above frame shift mutation in *chiA* lacks the genes for polyglutamate synthesis (*Dpga*), and (2) F11.4 which has the *Dpga* deletion and is completely lacking both of the chitinase encoding genes *DchiBA*¹⁶.

Here, we report findings that the two almost isogenic *B. licheniformis* strains F11.1 and *B. licheniformis* F11.4 display different protease production patterns and substrate specificities especially on collagen.

MATERIAL AND METHODS

Bacterial Strains and Growth Conditions

In this study we used *B. licheniformis* F11.1 (*DchiA*; *Dpga*) and *B. licheniformis* F11.4 (*DchiAB*; *Dpga*). For detailed description we refer to Waldeck *et al.*¹⁵. Two types of media were used for bacterial growth and protease production : Luria Broth (LB) and Modified Luria Broth plus collagen (MLB +collagen) media. The LB media contained tryptone 1%, NaCl 1% and yeast extract 0.5%. The Modified LB + collagen media contained tryptone 0.5%; NaCl 1%, yeast extract 0.25% and collagen 5%. Cell growth was monitored turbidimetrically by measuring the absorbance at $\lambda = 620$ nm. 10 % of the seedling culture with an OD₆₂₀ of 0.8 was inoculated into the same media for enzyme production. Incubation was performed at 37 °C and samples were taken at regular intervals for analysis of enzyme activity and cell growth.

Protein Assay and Protease Activity

The protein concentration was measured according to Bradford¹⁷ using reagents consisting of 100 mg Coomassie brilliant blue (CBB) G-250 in 50 ml 95 % ethanol and 100 ml 85 % phosphate acid in 1 liter. Bovine serum albumin served as the protein standard. Each experiment was done in triplicate.

Protease activity was measured according to the method proposed by Bergmeyer *et al.*¹⁸ using either casein, collagen, gelatin or fibrin at 1 % w/v concentration in buffer Tris-HCl 0.05 M. 50 μ l enzyme filtrate was mixed with 250 μ l substrate and incubated for 10 minutes at 37 °C. Trichloroacetic acid (TCA) 0.2 M was added and kept at 37 °C for 10 minutes followed by centrifugation (4000xg, 10 minutes). The supernatant was mixed with 0.4 M Na₂CO₃, followed by addition of Folin Ciocalteu reagent (1:2) and further incubation at 37 °C for 20 minutes. The reaction products were measured at $\lambda = 578$ nm. Substrate solution without enzyme was used as control. One unit (U) of enzyme activity equals 1 μ mole tyrosine per min.

Zymogram Analysis

For zymogram analysis, a modified SDS-PAGE procedure was applied. The polyacrylamide gel used were 8 % for separating gel and 4% for collecting (modified after Lantz and Ciborowski¹⁹. Acrylamide was copolymerized with 1% of the protein substrates. Following electrophoresis, the

gel was soaked in Triton X-100 2.5% for 1 hour and further incubated for enzyme substrate reaction in Tris-Cl 10 mM, pH 8 for 24 hours. The activity bands were visualized after (50 % methanol + 10 % acetic acid + 0,06 % Coomassie brilliant blue R-250) for 30 minutes followed by incubation in destaining solution (5 % methanol + 7,5 % acetic acid). Positive results corresponded to clear bands. Estimation of the molecular weight was conducted by using Low Molecular Weight protein standard phosphorylase b (97 kDa), BSA (bovine serum albumin) (66,0 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

RESULTS

The *Bacillus licheniformis* F11 showed high protease activity but lacking chitinase activities. Morphology characteristics was conducted previously and the F11 displayed a rough colony morphology, and motile with rods shape. Further physiological and biochemical characterization suggested representatives of the *Bacillus licheniformis* species. Definite evidence for the affiliation of the isolate with the species *B. licheniformis* was obtained from sequencing the 16S rRNA gene which indicated 100% identity to *B. Licheniformis* DSM13/ATCC 14580. The genetic analysis had included a

number of loci encoding extracellular degradative enzymes, including adjacent regions, and the *degS-degU* operon instrumental in the regulation of such enzymes, as well as the *pga* locus, which encodes the polyglutamic acid synthetase. When a Blast search was performed with the deduced polypeptides, 99 to 100% identity to known *Bacillus licheniformis* loci became evident, with only one exception, i.e., *chiA*¹⁵. By aligning the corresponding predicted proteins and DNA sequences it became evident that there is a frameshift mutation in *chiA* in F11.1 and there is another additional mutation in *chiB* in F11.4¹⁶. In this research, different enzyme expression and pattern from both isolates were obtained.

The strains *B. licheniformis* F11.1 and F11.4 differ genetically only with respect to their chitinase genes, the former has the frame shift mutation in *chiA* while the latter has the chitinase encoding genes *chiBA* completely deleted. The *Dpga* deletion is common to both mutants, it was introduced to save stirrer energy during fermentation (reduced viscosity) and no metabolic energy is wasted for an unwanted by-product. Since both strains performed rather differently in deproteinization experiments with shrimp shells^{15,16}. We were eager to learn more about their capacity to produce proteolytic enzymes.

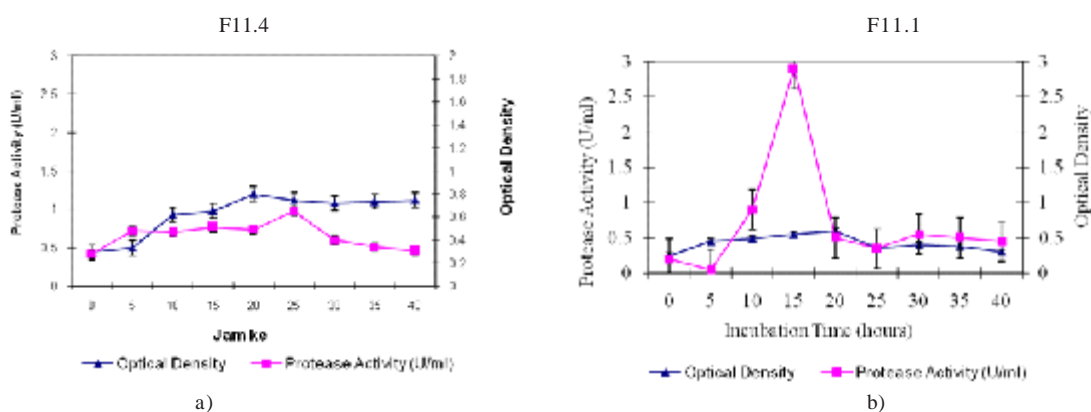


Fig. 1. Protease activity of *B.licheniformis* 11.4 (A) and *B.licheniformis* 11.1 (B). Protease activity was measured using collagen as the substrate and the cell growth was monitored at 620 nm

As seen as Figure 1 showed that *B.licheniformis* 11.1 produced higher protease activity compared to the *B. licheniformis* F11.4. The optimum production time was 15 for *B.*

licheniformis F11.1 with a protease activity of 0.028 U/ml. For *B. licheniformis* F11.4 it was between 20 - 35 hours of fermentation, with enzyme activity of 0.005 U/ml. After 15 hours in LB media,

the protease activity of *B. licheniformis* F11.1 decreased significantly. The decreasing enzyme activity might be due to autohydrolysis of the protease molecules. The lower protease activity of *B. licheniformis* F11.4 appeared to correlate with the more stable and longer period of appearance of enzyme activities.

Earlier study by Hoffmann *et al.*¹⁶ who used Luria Bertani media with azocasein as the substrate reported similar result, that is, *B. licheniformis* F11.1 showed shorter time for protease synthesis (20 hours), and *B. licheniformis* F11.4 required longer time of 40 hours to optimally

produced its protease. In LB media, the protease activity of *B. licheniformis* F11.1 was higher than that of extracellular *B. licheniformis* F11.4. The extracellular protease from *B. licheniformis* F11.1 was effective for deproteinization of the protein attached to the shrimp waste. The collagenase activity of *Bacillus licheniformis* F11.4 was reported²⁰. Addition of collagen into the media LB, resulted in different responses. In this case, the concentration of tryptone and yeast extract in the original LB media was reduced by half, and collagen was added at 5%, the activity of *Bacillus licheniformis* F11.4 was increased²⁰.

LB

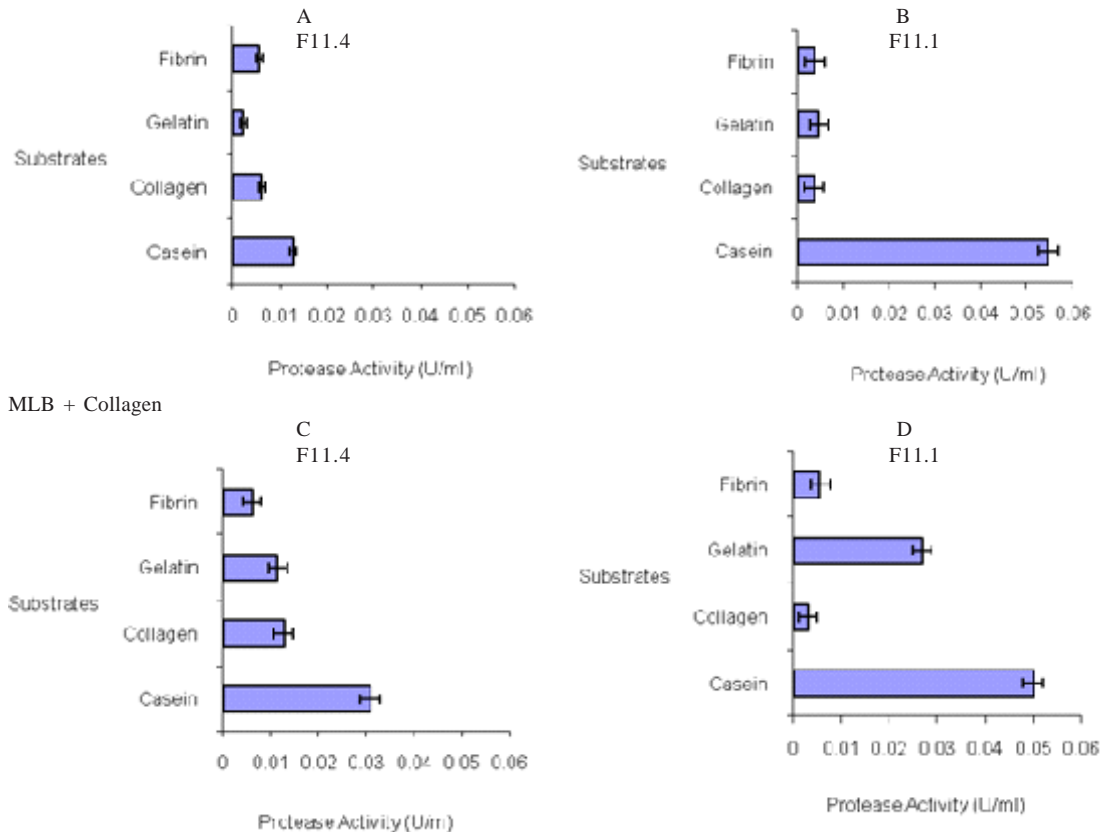


Fig. 2. Activity of protease from *Bacillus licheniformis* 11.4 (A and C) and *Bacillus licheniformis* 11.1 (B and D) produced in media LB (A and B) and media modified LB + Collagen (C and D)

Figure 2 showed difference pattern substrate preferences of protease from *B. licheniformis* F11.1 and *B. licheniformis* F11.4. When produced in LB media, both protease were more active towards casein compare with the other 3 protein substrates (collagen, gelatin and fibrin).

Furthermore, when gelatin was used as the substrate for enzyme analysis, the protease from *B. licheniformis* F11.4 showed higher enzyme activity up to 3 times higher than of *B. licheniformis* F11.1. Addition of collagen into the media induced higher synthesis of fraction

capable of degrading collagen. Zymography analysis in collagen gel (Figure 3) showed increase

not only in the number of clearing band but also more intense clearing band.

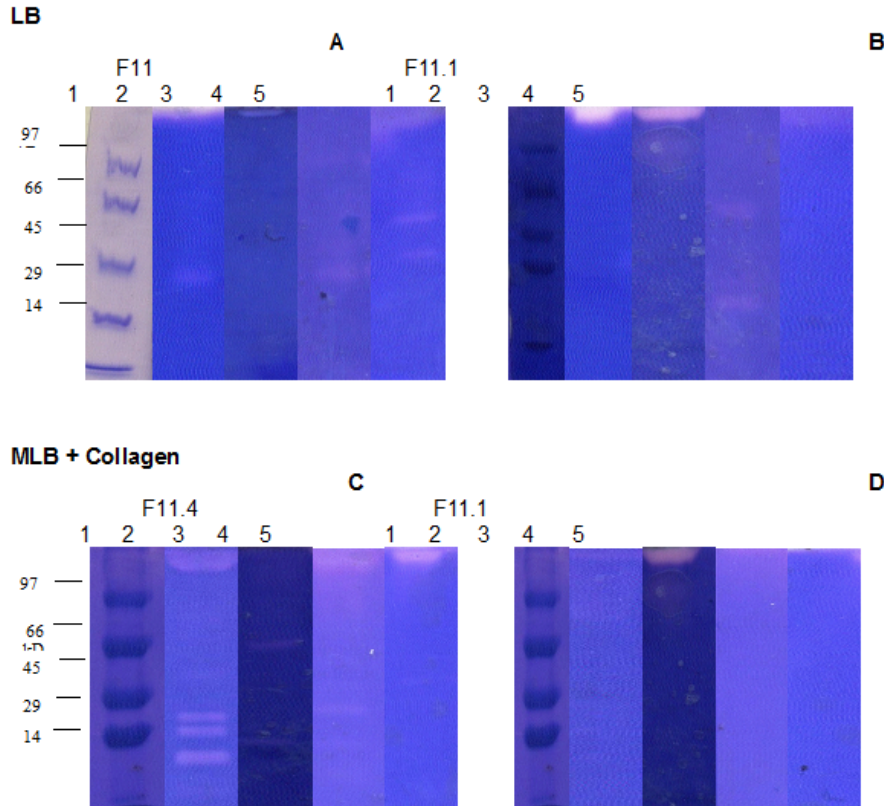


Fig. 3. Activities of protease fractions from *B. licheiformis* F11.4 (A and C) and *B. licheniformis* F11.1 (B and D) on different protein substrates. The protease was produced using LB media (A and B) and MLB + Collagen media (C and D). Zymogram analysis was conducted using collagen (line 2), casein (line 3), gelatin (line 4) and fibrin (line 5). Line 1 indicated protein marker.

Figure 3 and Table 1 confirmed the different protease fractions produced by both *B. licheiformis* F11 mutants grown in different media. Diversity of the protease fractions with different apparent molecular weight were detected through zymography analysis. *B. licheniformis* F 11.4 synthesized protease fraction with higher

molecular weight on the four substrates tested (casein, collagen, gelatin and fibrin). Table 1 also indicated that the protease fraction produced by *B.licheniformis* F11.4 in the collagen containing media were more active toward not only collagen but to other protein substrates as well.

Table 1. Summary of protease fractions produced by *B. licheiformis* F11.4 and *B. licheiformis* F11.1 detected in zymogram analysis

MW	F11.4				F11.1			
	Casein	Collagen	Gelatin	Fibrin	Casein	Collagen	Gelatin	Fibrin
< 40	√		√	√				
40-90	√	√	√	√				
>90		√	√	√	√	√	√	√

DISCUSSION

The optimum production time was 15 hours for *B. licheniformis* F11.1 and 12-35 hours for *B. licheniformis* F11.4. The higher enzyme activity might be responsible to the enzyme autohydrolysis which is shown as quick reduction in the enzyme activity following it is optimum level. This is different from the protease from *B. licheniformis* F11.4 which remained high in the LB media at longer time intervals. For *B. licheniformis* F11.1, the optimum enzyme was produced during logarithmic phase and also reached maximum activity at the time close to stationary phase.

Generally, extracellular proteases are produced by *Bacillus* sp. during post exponential and stationary phases. Little or no enzyme production had been reported during exponential growth phase of *Bacillus*; however protease production in *B. subtilis* ATCC14416 and *B. sphaericus* BSE 18 occurs at mid-exponential phase, and rapid auto deactivation was observed after the culture reached maximum enzyme activity. There is case which reported that exponential growth phase was the phase in which the synthesis and secretion of the protease was initiated, with a substantial increase near the end of the growth phase and maximum amounts of protease produced in the stationary growth phase²¹.

The optimum fermentation time for synthesis of a particular enzyme is affected not only by different bacterial strain but also by varieties of fermentation conditions. In general, the *Bacillus subtilis* CN2 was reported to synthesize protease optimally by 14 hours of fermentation time⁶. Shorter optimum fermentation time of protease, ie. 9 hours was reported when *Bacillus* SMIA-2 was grown in media containing trisodium citrate 1%⁷. *Bacillus subtilis* PE-11 produced protease optimally at longer incubation time, that by 48 hours when grown in nutrient broth⁸.

In collagen media, the extracellular protease from both *B. licheniformis* were more active toward collagen eventough casein was still best substrate. Peptide bond in casein structure are more accessible for enzyme hydrolysis, while more complex three dimensional fibril structure of other substrates contribute to reduced chance of enzyme substrate interaction. Due to their triple

helical nature, collagen and gelatin is usually resistant to many general proteases²¹. Protease from *Bacillus* also showed preferences to protein substrate like casein, albumin and less active to the more complex protein such as collagen, keratine and fibrin²².

Excretion of several protease molecules which are capable of degrading different substrates is a general phenomenon found in many bacteria and microorganisms. This is essential to utilize the numerous protein substrates found in nature^{1,2}. Nowadays, multi proteases which are able to degrade different protein substrates such as gluten and casein is much applied as agents to help indigestion problems. In our study, beside capable of digesting casein and collagen, protease of *Bacillus licheniformis* F 11.1 and F 11.4 could hydrolyse gelatin and fibrin. Another protein which was found as substrate for the enzymes was wheat derived gluten known as protein which cause alergenicity (data not shown). Protease treatment is one of the many proposed techniques useful to reduce some allergenic responses.

CONCLUSIONS

The presence of collagen in the production media clearly induced enzyme fractions which were more active towards collagen. Zimography assay is thus useful to get more information on the enzyme molecular fractions synthesized at various conditions. The different genetic mutation of the same species (*Bacillus licheniformis* F11.1 and F11.4) results in different metabolic activities which result in difference characteristic of the protease molecules synthesized.

ACKNOWLEDGEMENTS

This research was funded by Indonesian Ministry of Education and Culture through 2010 International Research Scheme. *Bacillus licheniformis* F11 used in this research were the result of research collaboration between Indonesia (Agency for the Assessment and Application of Technology Jakarta) and German (Indo-German Biotechnology). We gratefully acknowledge for Prof. Dr. Friedhelm Meinhardt and his colleague for constructed of the mutant *Bacillus licheniformis* F11.1 and F11.4

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