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Selection of Methods for Microbiological Extraction of Chitin from Shrimp Shells

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Chitin extraction from shrimp shells involves two processing steps that are demineralization followed by deproteination process. *Lactobacillus acidophilus* FNCC 116 and *Bacillus licheniformis* F11.1 were used in demineralization and deproteination respectively. The overall objectives of this experiment were to determine fermentation systems which resulted in the highest mineral and protein removal. The demineralization experiments consisted of three different batch fermentation designs: batch fermentation (A_m); subsequent batch fermentation 1, in which 100% medium was replaced with fresh medium after 24 h fermentation (B_m); and subsequent batch fermentation 2, in which 50% medium was replaced with the same amount of fresh medium after 24 h fermentation (C_m). The demineralization was conducted at 30±2 °C, 50 rpm for 60 h. The deproteination experiments consisted of 3 different batch fermentation designs: batch fermentation 1, inoculum was added once at the beginning of the fermentation (A_p); batch fermentation 2, inoculum was added twice, at the beginning and after 24 h fermentation (B_p); and subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation (C_p). The deproteination was carried out at 55 °C, pH 7.8-8.0, aeration 2.3 vvm and agitation 275 rpm for 96 h. The experimental results showed that in the demineralization process, fermentation design B_m gave the highest ash removal. Ash removed in the fermentation design A_m, B_m, and C_m was 97.19, 99.69, and 97.69% respectively. The protein removed in the fermentation design A_p, B_p, and C_p was 94.42, 94.51, and 95.37% respectively.

Key words: demineralization, deproteination, microbiological chitin extraction, shrimp shells

Ekstraksi kitin dari kulit udang terdiri atas dua tahap proses yaitu proses demineralisasi dilanjutkan dengan deproteinasi. *Lactobacillus acidophilus* FNCC 116 dan *Bacillus licheniformis* F11.1 masing-masing digunakan untuk proses demineralisasi dan deproteinasi. Tujuan penelitian ini adalah untuk menentukan sistem fermentasi yang dapat menghilangkan mineral dan protein dari kulit udang terbanyak. Percobaan demineralisasi terdiri atas 3 rancangan fermentasi sistem tumpak yang berbeda: fermentasi sistem tumpak (A_m); fermentasi sistem tumpak berurutan 1, di mana 100% medium diganti dengan medium segar sesudah fermentasi 24 jam (B_m); dan fermentasi sistem tumpak berurutan 2, di mana 50% medium diganti dengan medium segar dalam jumlah yang sama sesudah fermentasi 24 jam (C_m). Proses demineralisasi dilakukan pada 30±2°C, 50 rpm selama 60 jam. Percobaan deproteinasi terdiri atas 3 rancangan proses yang berbeda: fermentasi sistem tumpak (A_p), inokulum ditambahkan sekali pada awal fermentasi; fermentasi sistem tumpak 2 (B_p), inokulum ditambahkan 2 kali, pada awal dan setelah fermentasi 24 jam; fermentasi sistem tumpak berurutan: 100% medium diganti dengan medium segar setelah 24 jam (C_p). Proses deproteinasi dilakukan pada 55 °C, pH 7,8-8,0, aerasi 2,3 vvm dan agitasi 275 rpm selama 96 jam. Hasil percobaan menunjukkan bahwa dalam proses demineralisasi, rancangan fermentasi B_m dapat menghilangkan kadar abu terbanyak. Kadar abu yang dihilangkan dalam rancangan fermentasi A_m, B_m, dan C_m masing-masing 97,19%, 99,69 %, dan 97,69%. Pada proses deproteinasi, protein yang dihilangkan dari rancangan fermentasi A_p, B_p, dan C_p masing-masing 94,42%, 94,51%, dan 95,37%.

Kata kunci: demineralisasi, deproteinasi, ekstraksi kitin secara mikrobiologi, kulit udang

Chitin is a homopolymer of N-acetyl-D-glucosamine residues linked by β-1, 4 bonds. Chitin and its derivatives have many applications in food, pharmaceutical, photography, cosmetic, paper, and textile industries (Dutta *et al.* 2004). Chitin can be found in various animals and plants and the crustacean is the most important chitin source for commercial use. In crustacean waste chitin is associated with protein, minerals, especially calcium carbonate, lipids, and pigments (Mahmoud *et al.* 2007). Consequently, to extract chitin from crustacean waste will involve removal of the protein (deproteination), minerals (demineralization), lipids, and pigments. Conventional chitin extraction used harsh chemicals that caused environmental pollution, since concentrated hydrochloric acid was used for demineralization and sodium

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hydroxide for deproteination. Some studies have been done to reduce or even to eliminate the environmental problems (Healy *et al.* 2003; Beaney *et al.* 2005; Rao and Stevens 2005; Jung *et al.*2006; and Prameela *et al.*2010). These studies include deproteination of the crustacean shells using proteolytic enzyme or microorganisms. Beaney *et al.* (2005) showed that the functional properties of the bioprocessed chitin were similar to those of chemically extracted chitin. Microbiological demineralization and deproteination have been done by Healy *et al.* (2003), Rao and Stevens (2005), Jung *et al.* (2006), and Prameela *et al.* (2010).

Based on our previous study, demineralization prior to deproteination in chitin extraction resulted in better ash and protein removal (Wahyuntari *et al.* 2011). The objective of trying different demineralization experiments was to find the optimum lactic acid production in order to get maximum ash removal. Parameters observed in this experiment were growth of *Lactobacillus acidophilus* FNCC 116, glucose consumed by the bacteria during fermentation, lactic acid produced, and pH course during fermentation. Three different deproteination experiments were done to optimize protease production for hydrolyzing protein in the shrimp shells. The parameters studied were bacterial growth of *Bacillus licheniformis* F11.1, protease produced and protein content of the protease supernatant (cell free fermented broth).

MATERIAL AND METHODS

Shrimp Shells and Microorganisms. Headless shrimp shells of *Penaeus vannamei* were obtained from frozen shrimp processing company "PT Wirontono Baru" North Jakarta, Indonesia. The shells were washed and disintegrated into 5-10 mm size, kept in -20 °C before using it for experiments.

L. acidophilus FNCC 116, a lactic acid producing bacterium was used for demineralization process. The bacterium was obtained from Food Nutrition Culture Collection of Faculty of Agicultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The stock cultures in 10% glycerol and 10% skimmed milk were stored in deep freezer at -80 °C.

B. licheniformis F.11-1 used for deproteination process of shrimp shells was isolated from shrimp shell waste of PT Lauraindo, a frozen shrimp processing company, Palembang, Sumatera, Indonesia. The bacterium was isolated and identified by Waldeck et al (2006) and molecularly modified by Hoffman *et al* (2010). The stock culture was kept in 10% glycerol and 10% skimmed milk at -80 °C.

Demineralization Process. Completely randomized design was used in experiment of shrimp shell demineralization. The demineralization experiments consisted of 3 different batch fermentation designs: batch fermentation (A_m); subsequent batch fermentation 1 (B_m), 100% medium was replaced with fresh medium after 24 h fermentation (the fermented medium was centrifuged to separate the cells and shrimp shells waste, the supernatant was discarded and replaced with fresh medium); subsequent batch fermentation 2 (C_m), in which 50% medium was replaced with the same amount of fresh medium after 24 h fermentation. The demineralization was conducted in 2 L fermentor (contained 1 L medium) at 30±2 °C, 50 rpm for 60 h. Refreshing frozen stock culture was conducted by transferring 1 mL stock culture into 9 mL of sterile de Man Rogosa Sharpe (MRS) broth and incubated at 37 ºC for 24 h. To prepare a starter inoculum, 10 mL refreshed culture was transferred into 90 mL MRS broth in 250 mL Erlenmeyer flask and incubated at 37 °C until its optical density reached 0.85 at wavelength of 600 nm with cell concentration about $1x10^9$ CFU mL^{-1}. Based on previous study optimum demineralization condition was at ambient temperature (30 ± 2 °C) and 50 rpm agitation (Junianto *et al.* 2009). Three hundred grams frozen shrimp shell waste (69.5% moisture) were added with 900 mL liquid media and 100 mL starter inoculums. In 100 mL medium contained 6 g glucose, and 0.05 g yeast extract, pH was adjusted to pH 7. The fermentation was done at 37 °C and 50 rpm agitation for 48 h. Each experiment was repeated twice. When demineralization process was completed, the shells were separated from the broth and washed with running water until the washed water became neutral (pH 7) and drained. The demineralized shells then were kept in -20 °C for the following process.

Deproteination Process. Completely randomized design was used in the deproteination experiment of demineralized shrimp shells. The deproteination experiments consisted of 3 different batch fermentation designs: batch fermentation 1 (A_p), inoculumn was added once at the beginning of the fermentation; batch fermentation 2 (B_p), inoculum was added twice, at the the beginning and after 24 h fermentation; subsequent batch fermentation (C_p), 100% medium was replaced with fresh medium after 24 h fermentation (the fermented medium was centrifuged to separate the cells and shrimp shells waste, the supernatant was discarded and replaced with fresh medium). The frozen stock

culture of *B. licheniformis* F11.1 was refreshed in Luria broth media. The culture was incubated in shaker incubator at 55 °C, 180 rpm for 6 h or until the optical density of the culture reached 0.9, which based on previous experiment the cell density was equal to 1×10^9 CFU mL^{-1} (Junianto *et al.* 2009). Each 100 mL fermentation medium contained 0.5 g KH_2PO_4, 0.5 g NaCl, 0.5 g yeast extract, 0.05 g $MgSO_4$, and 0.1 g $CaCl_2$. Two hundreds mL inoculum was added into 300 g of shrimp shells in 800 mL medium. The fermentation was carried out at 55 °C, 2.5 vvm aeration, 275 rpm agitation for 60 h and the pH was maintained at the range of 7.8-8.2. Samples for analytical assays were taken every 6 h. After the deproteination process completed, the shells were separated from the broth, washed, drained, and kept at 20 °C for the following process.

All fermentations were conducted in custom made fermentor consisted of 2 L glass cylinder jar equipped with Jake and Kunkel rod agitator for agitation, a compressor connected to LKB-Bromma, flow meter for aeration and heated water in water bath that was being circulated using coil into the jar for temperature control. The shrimp shells were not sterilized prior to any fermentation.

Analytical Procedures. Demineralization process of the shrimp shells (before, during, and after fermentation), moisture, ash, and insoluble protein content of the shrimp shells were observed, whereas the parameters observed of the fermented broth in demineralization process were bacterial density, glucose, lactic acid, and pH. Parameters observed during deproteination process were bacterial density and protease production in the fermented broth, as well as protein content of the shrimp shells. Moisture content was determined by heating samples at 110 °C in "Kett" infrared moisterure meter model F-1A (Tokyo, Japan). Ash content was determined after combustion of 5 g dried sample in a crucible at 600 °C for 4 h in muffle furnace (AOAC 1984). Insoluble protein content of the shrimp shells and fermented solid sample was solubilized using 1M NaOH. Seven poin five (7.5) mL of 1M NaOH was added to 0.5 g sample and then incubated for 24 h. The protein content of the supernatant was measured according to Lowry *et al.* (1951) method using bovine serum albumin fraction IV (Sigma) as a standard.

Glucose and lactic acid content was analyzed using HPLC (Merck-Hitachi), Aminex column HPX-87H (300mm × 7.8mm), at 65 °C (L-5025-Column Thermostat), isocratic mobile phase of 0,005 N H_2SO_4 with flow rate of 0.6 mL min^{-1} (L-6200A-Pump, Merck-Hitachi), differential refractometer detector RI-71 (Merck). Glucose standard used 1% glucose (Sigma-Aldrich) and lactic acid standard was 10% L-lactic acid (Oxoid).

Protease activity in fermented broth was assayed using azocasein as a substrate according to the method described by Waldeck *et al.* (2006). One unit was defined as the amount of enzyme releasing 1 mol azocasein per min under reaction conditions. The density of bacterial growth in fermentation broth was assayed after serial dilution by counting colony forming unit (CFU mL^{-1}) on MRS agar plate after incubation at 37 °C, 24 h for *L. acidophilus* FNCC116 and on Luria Bertani agar plate after incubation at 55 °C, 24 h for *B. licheniformis* F11.1.

Decrease of the ash and protein content in shrimp shells were analyzed statistically using F test and Duncan's multiple range tests at 95% confidence. Experimental results of other parameters were descriptively analyzed in the graphic forms.

Chitin concentration of the demineralized and deproteinized shrimp shells was calculated based on equation 1, according to Mizani and Aminlari (2007).

$$\text{Chitin (\%)} = \text{chitin nitrogen} \times 14.25 \quad (1)$$

Total nitrogen of the demineralized and deproteinized shrimp shells (chitin) was analysed using Kjeldhal method (AOAC 1984).

RESULTS

Demineralization. Bacterial growth in batch fermentation A_m reached the maximum growth (1.96×10^9 CFU mL^{-1}) after 24 h fermentation, and then started decreasing afterward until the end of observation (2.53×10^8 CFU after 60 h) (Fig 1). Maximum bacterial growth in fermentation B_m was also reached after 24 h (1.81×10^9 CFU). Eighteen h after replacing the medium or 42 h from the beginning of fermentation, the cell amount reached 2.07×10^9 CFU, and then the cell amount decreased to 5.78×10^8 CFU at the end of fermentation (60 h observation) (Fig 1). In the second subsequent batch C_m, the maximum cell growth reached 1.99×10^9 CFU after 24 h. After replacing 50% medium with the fresh one, the cells reached maximum amount of 1.55×10^9 CFU after 12 h or after 36 h since the beginning of fermentation, then the cell amount decreased to 5.68×10^8 CFU at the end of observation (Fig 1).

Initial glucose concentration was 6% (w/v), and after 24 h fermentation the remaining concentration of the

glucose of all fermentation design was about 1% (w/v) (Fig 2). In fermentation B_m and C_m, the medium was replaced after 24 h with a 100% and 50% fresh medium, thus there was glucose addition. The rapid decrease of glucose concentration in the first 6 to 24 h afterward (Fig 2) corresponded to the increase of bacterial cells (Fig 1). After 48 h fermentation, glucose in the medium was continuously consumed until the end of the observation, where less than 2% glucose remained.

Lactic acid produced in all fermentation designs after the first 24 h of fermentation was about the same which was 2.17, 2.30, and 2.26%, repectively. After that, lactic acid concentration in batch fermentation A_m did not increase. However, the lactic acid concentration in fermentation B_m and C_m increased after fresh medium addition. The highest lactic concentration was produced in B_m (4.19%) and in C_m (3.42%) (Fig 3).

The pH value of the fermentation broth depended on the lactic acid concentration. The lowest pH was achieved in fermentation B_m (pH 3.88) and the highest pH in A_m (pH 4.61) (Fig 4). The ash concentration in the shell in all treatments decreased rapidly for the first 24 h, and then the ash removal was slowing down until the end of observation. The ash concentration of the shells in A_m, B_m, and C_m was 0.55, 0.06, and 0.45% which represented 97.19, 99.69, and 97.69% ash removed respectively (Fig 5).

Deproteination. Maximum bacterial growth of all fermentation systems was reached after 12 h incubation, and then started declining for the following 12 h, in fermentation A_p continuously declining until the end of observation. After addition of inoculum in fermentation B_p and replacement of medium in C_p, the cell amount started increasing. The cell amount in B_p started declining 6 h after addition of inoculum and in C_p declined gadually for the following 12 h and decreased fast for the last 6 h of observation (Fig 6).

Maximum production of protease during deproteination in A_p, B_p and C_p was reached after 48, 72, and 72 h incubation respectively. The highest enzyme activity was 16.51 U mL^{-1} in B_p, the second was 16.06 U mL^{-1} in A_p, and the lowest was 11.63 U mL^{-1} in C_p (Fig 7). Protein concentration of the shrimp shells in fermentation system A_m, B_m, and C_m 5.58, 5.49, and 4.63% respectively which represented 94.42, 94.51, and 95.37% protein removed. Aproximate analysis of chitin produced by combination of demineralization B_m and deproteination A_p showed as follows: 0.84% ash, 1.46% protein, and 97.85% chitin (% dry base).

DISCUSION

Based on our previous report, in the microbiological chitin extraction from shrimp shells, demineralization prior to deproteination gave a better chitin yield (Wahyuntari *et al.* 2011), therefore in this experiment, demineralization was carried out before deproteination process.

Demineralization. Fermentation system of *L. acidophilus* FNCC 116 used for lactic acid production in this experiment was batch and modification of fed batch. Batch, fed batch, or modification of both systems are common fermentation system used in industries (Hsu and Wu 2002). The efficiency of lactic

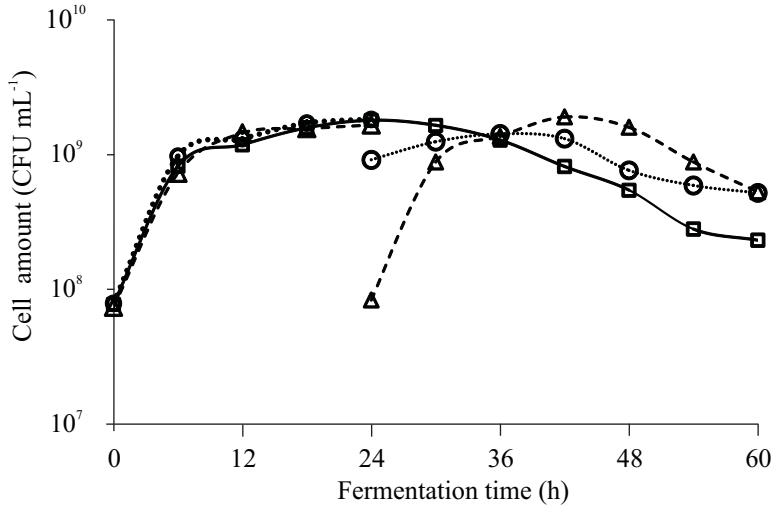

Fig 1 Growth of *Lactobacillus acidophilus* FNCC 116 in different fermentation systems. A_m (□): batch fermentation; B_m (△): subsequent batch fermentation, in which 100% medium was replaced with fresh medium after 24 h fermentation; C_m (○): subsequent batch fermentation, in which 50% medium was replaced with the same amount of fresh medium after 24 h fermentation.

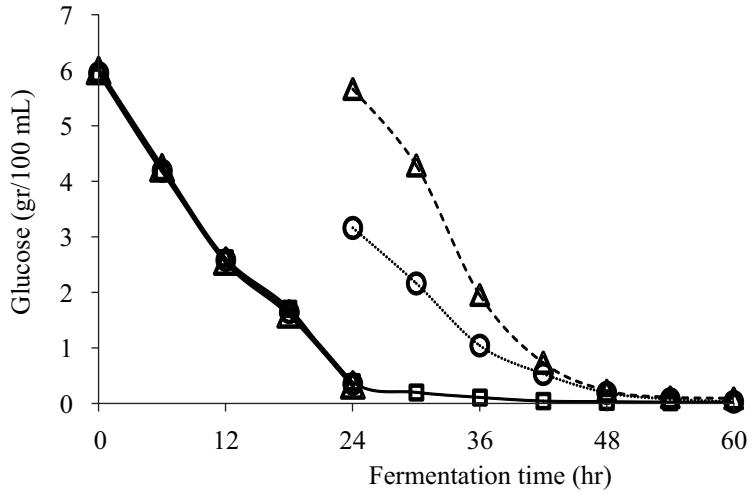

Fig 2 Glucose consumption during demineralization of shrimp shells by *Lactobacillus acidophilus* FNCC 116 in different fermentation systems. A_m(□): Batch fermentation; B_m (△): Subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation; C_m (○): Subsequent batch fermentation, 50% medium was replaced with the same amount of fresh medium after 24 h fermentation.

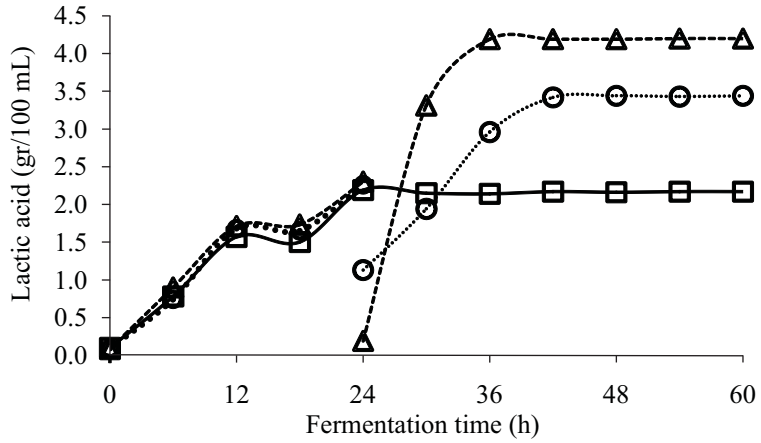

Fig 3 Lactic acid production during demineralization of shrimp shells by *Lactobacillus acidophilus* FNCC 116 in different fermentation systems. A_m(□): Batch fermentation; B_m (△): Subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation; C_m (○): Subsequent batch fermentation, 50% medium was replaced with the same amount of fresh medium after 24 h fermentation.

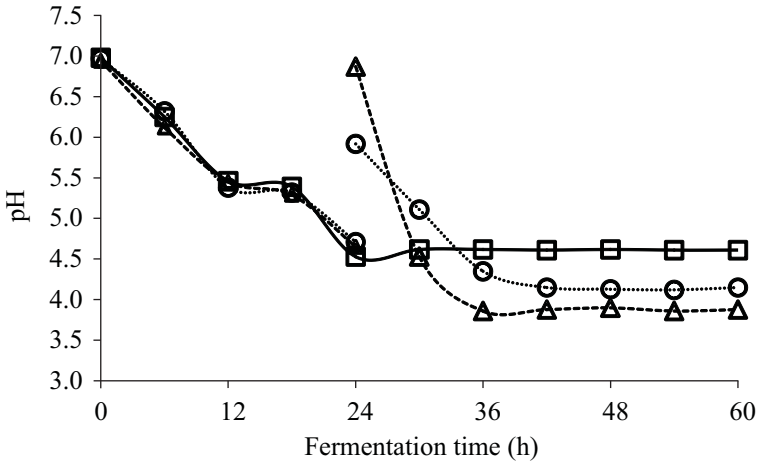

Fig 4 pH of fermented broth during demineralization of shrimp shells by *Lactobacillus acidophilus* FNCC 116 in different fermentation systems. A_m(□): Batch fermentation; B_m (△): Subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation; C_m (○): Subsequent batch fermentation, 50% medium was replaced with the same amount of fresh medium after 24 h fermentation.

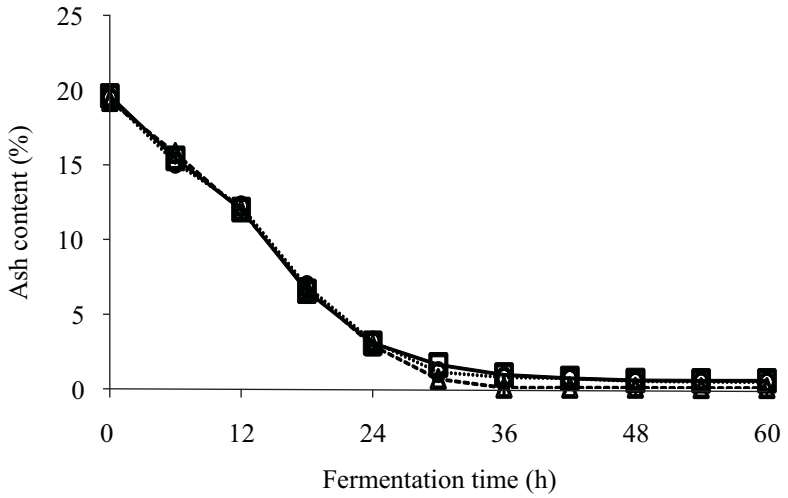

Fig 5 Ash content in shrimp shells during demineralization of shrimp shells by *Lactobacillus acidophilus* FNCC 116 in different fermentation systems. A_m(□): batch fermentation; B_m (△): subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation; C_m (○): subsequent batch fermentation, 50% medium was replaced with the same amount of fresh medium after 24 h fermentation.

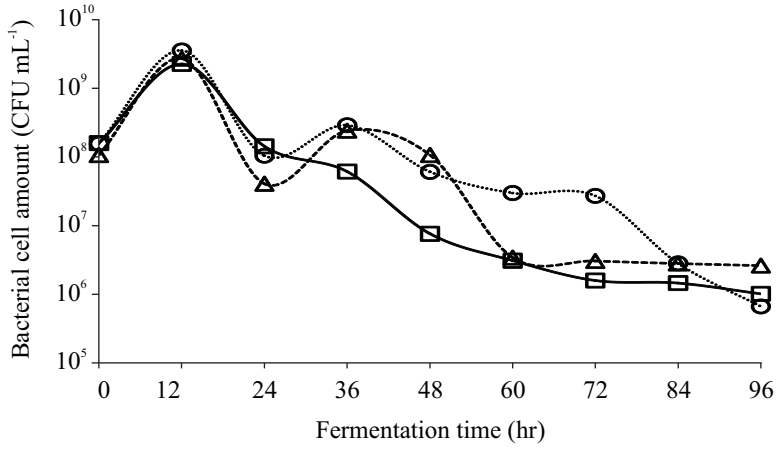

Fig 6 Growth of *Bacillus licheniformis* F.11.1 during deproteination of shrimp shells. A_p (□): batch fermentation 1, inoculum was added once at the beginning of the fermentaion; B_p(△): batch fermentation 2, inoculum was added twice, at the beginning and after 24 h fermentation; C_p (○): subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation.

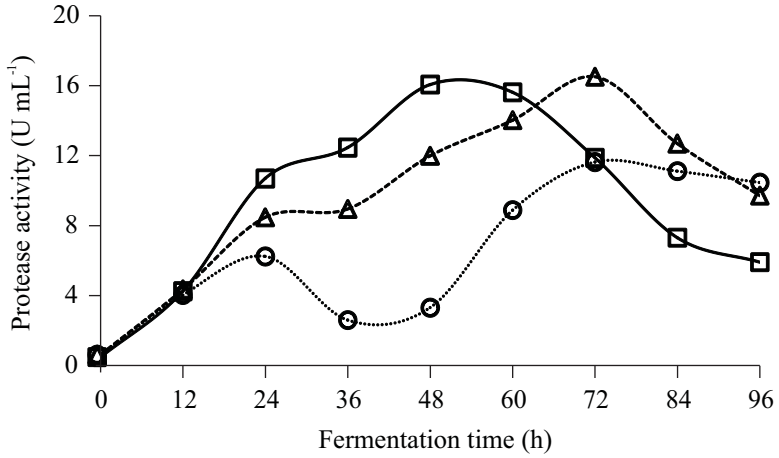

Fig 7 Protease production during deproteination of shrimp shells using *Bacillus licheniformis* F11.1. A_p (□): batch fermentation 1, inoculum was added once at the beginning of the fermentation; B_p(△): batch fermentation 2, inoculum was added twice, at the beginning and after 24 h fermentation; C_p (○): subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation.

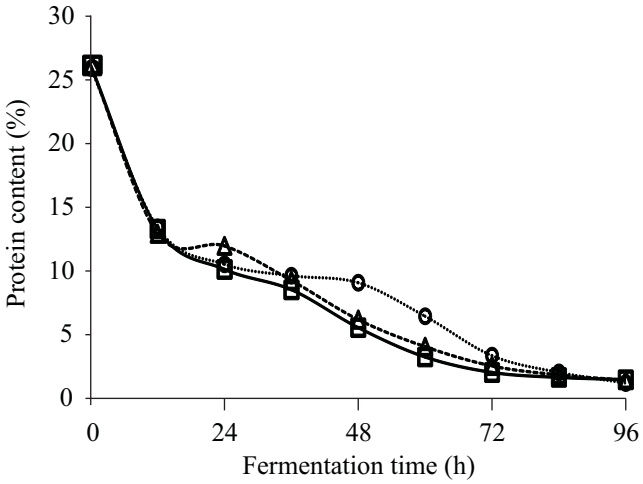

Fig 8 Protein content of shrimp shells during deproteination using *Bacillus licheniformis* F11.1. A_p (□): batch fermentation 1, inoculumn was added once at the beginning of the fermentaion; B_p(△): batch fermentation 2, inoculumn was added twice, at the beginning and after 24 h fermentation; C_p (○): subsequent batch fermentation, 100% medium was replaced with fresh medium after 24 h fermentation.

acid fermentation depends on some factors such as inoculum density, glucose concentration, and initial pH, during fermentation as well as fermentation time (Rao *et al.* 2000). *L. acidophilus* is a homofermentative lactic acid bacterium which produces lactic acid as a major product from glucose (Jafarei and Ebrahimi 2011). Based on experimental data, the bacterial exponential growth was associated with the glucose consumed and the lactic acid produced. In this demineralization experiment, initial pH and glucose concentration in the medium used in all batch process was pH 7 and 6%, respectively, with cell density of bacterial starter 8.5×10^8 CFU mL^{-1}. The effect of glucose concentration in the medium on lactic acid production was observed by applying three different fermentation schemes (fermentation A_m, B_m, and C_m). The aim of applying three different fermentations of lactic acid bacteria was to find the optimum lactic acid production. Since the lactic acid produced would react with calcium carbonate in chitin fraction of the shrimp shells to form calcium lactate which precipitate and can be removed by washing (Rao and Stevens 2005). Therefore, the more lactic acid produced, as expected the more calcium carbonate in the shrimp shells could be removed. Our proximate analysis showed that Calcium content of our sample was 39.21% of the ash content. The calcium removed was observed by analyzing the ash content of the shells, thus the more calcium removed the less ash content of the shells.

Experimental data showed that exponential growth phase of *L. acidophilus* FNCC116 lasted for 6 h for all batch system (A_m, B_m, and C_m) (Fig 1). After the first 6 h of fermentation, the bacteria grew exponentialy in batch A_m followed by stationary phase until 36 h incubation. When 100% medium was replaced with the fresh one in B_m, the cell undergone exponential phase again from 24 h to 36 h incubation. However, the maximum cell amount between A_m and B_m was not much different. When the medium was only replaced 50% (C_m), the cells did not grow as much as in B_m. In the exponential phase, the cells consumed most of the nutrients in the medium and in this experiment, the change of glucose concentration in the medium was observed. In the first 24 h, glucose concentration declined very rapidly in all fermentation system from 6% (w/v) and down to < 1% (w/v). After replacement of the medium, the glucose concentration returned to the original concentration in B_m since the medium was 100% replaced with the fresh one, whereas in C_m the medium was only replaced 50%, the glucose concentration became 3.16% (w/v) (Fig 2). After 48 h of incubation until the end of observation (60 h) the glucose concentration barely changed (< 0.2%).

Glucose consumed by the bacteria (Fig 2) was associated with lactic acid production (Fig 3). Lactic acid concentration increased rapidly up to 2.19-2.3% in the first 24 h in all fermentation systems which were related with the decrease of glucose concentration (Fig 2) and pH (Fig 4) in the medium. The data show that *L. acidhophilus* FNCC 116 converted some of glucose to lactic acid. Maximum concentration of lactic acid reached in A_m was 2.19% (w/v) and it did not change until the end of observation (60 h). Among the three fermentation systems, the highest lactic acid was in the system with 100% medium replacement (B_m). The highest lactic acid concentration was 4.19% (w/v) 12 h

after the medium replacement and the concentration remained at that value (4.2% w/v) until the end of observation (60 h) (Fig 3). In fermentation system C_m, where only 50% of the medium was replaced, the maximum lactic acid concentration was only 3.44% (w/v) (Fig 3). These data indicate that glucose added into the medium was mostly converted to lactic acid. Prameela *et al* (2010) study on chitin extraction using *L. plantarum* (ATCC 8014 and NRRL B-14768)*, L. pentosus* (ATCC 8041), and *L. fermentum* (NRRL B 1840) showed that the more glucose added the more lactic acid produced. The lactic acid produced affected the pH of the medium as (Fig 4). The initial pH of all system was 6.97-6.98. During the first 24 h, the pH of the medium decreased drastically to 4.53, 4.65, and 4.71 in A_m, B_m, and C_m, respectively. After 30 h, the lowest pH was in B_m (pH 3.88). In A_m the pH was 4.61. The pH value stayed the same during 60 h observation. However, in C_m after 30 h the pH of the medium was 4.35 and slightly decreased to 4.15 after 60 h observation pH value reached in fermentation B_m pH 3.88±0.00 during 60 h incubation with total glucose added 12%, this pH was lower than that in Prameela study (Prameela *et al.* 2010), were pH 4.173±0.01 was reached using *L. plantarum* NRRL B-14768 after 72 h fermentation with 15% initial glucose added. Jung *et al* (2005) reported that the pH value (pH 3.52±0.15) of fermentation using *L. paracasei* KCTC-3074 for 3 d (72 h) was slightly lower than our study (pH 3.88±0.00). The glucose added in Jung's study was 10% (Jung *et al.* 2005).

Bacterial growth, conversion of glucose to lactic acid and change of pH medium affected ash content of the shrimp shells (Fig 5). For the first 24 h of fermentation, ash content declined rapidly from initial concentration of 19.26-19.61% (w/v) down to 2.9-3.18% (w/v) with reduction rate of the ash concentration in the range of 0.034-0.035% (w/v) per hour. The reduction rate of the ash content in the shrimp shells was slowing down afterward until the end of observation (60 h) in the range of 0.022-0.027% (w/v) per hour. The highest ash reduction of the shrimp shells was in B_m (99.69%), followed by C_m (97.69%) and the lowest was in A_m (97.17%). Therefore, the lowest ash content of the shrimp shells after 60 h incubation was in fermentation B_m (0.06 ± 0.01% dry base) followed by C_m (0.45 ± 0.02% dry base) and A_m (0.55 ± 0.06% dry base). Based on Duncan Multiple range test, in demineralization process B_m, there was no significant difference in reduction rate of ash content in the shrimp shells after 24, 36, 42, 48, 54, and 60 h incubation. Based on the experimental data, demineralization process of shrimp shells suggested was the fermentation B_m with 36 h incubation, which was subsequent batch with 100% medium replacement after 24 h. The ash content in B_m after 36 h incubation was 0.08% ± 0.00% (dry base) or 99.61% ± 0.01% ash removed from the shrimp shells. The results of this demineralization process was better than previous study done by Healy *et al* (2003), Rao and Stevens (2005), Daum *et al.* (2007), and Prameela *et al.* (2012). Healy *et al* (2003) used a mixture of bacteria (*L. plantarum*, *L. salivarius*, *Streptococcus faecium*, and *Pediococcus acidilacti*) to demineralize and deproteinize prawn *(Nephros norvegicus)* at 30 °C for 7 d, and the final ash removed from the shells was 87.9%. Rao and Stevens (2005), used *L. plantarum* 541 for demineralization and deproteination, 63% ash 66% protein were removed. Daum *et al* (2007) used shells of *Penaeus* sp and *Lactobacillus sp* for demineralization process at 37 °C for 3 d, with 8% glucose added, 10% inoculum (10^8 cells mL^{-1}), resulted in 89.6% ash removed. Prameela *et al* (2012) used *L. plantarum* NRRL B-14768 with 15% glucose added, the fermentation was done at 37 °C for 72 h resulting 76% ash, and 89% protein removed.

Deproteination. Deproteination of demineralized shrimp shells was done by cultivating protease producing bacterium *B. licheniformis* F11.1. Three different fermentation systems were applied to optimize protease production for hydrolyzing the protein which associated with the shrimps shells.

In the system A_p, after 12 h incubation, the bacterial cells amount declined until the end of observation (96 h). In system B_p the cells amount decreased from 12 h to 24 h, then after addition of medium the cell amount increased again until 36 h incubation, then decreased again until the end of observation (96 h). In system C_p, after 12 h incubation, the cell amount decreased until 24 h of incubation. After addition of inculum after 24 h, the cells amount increased for 12 h (36 h incubation) then started decreasing until the end of incubation (96 h). Among the three fermentation systems, the least cell amount declining rate was in C_p.

The highest protease production in A_p (16.06 ±0.03 U mL^{-1}) was reached after 48 h incubation, in B_p was after 72 h (16.51±0.01 U mL^{-1}) and in C_p was after 72 h (11.63±0.04 U mL^{-1}). Addition of inoculum after 24 h (B_p) only slightly increased the protease production but delayed the maximum production from 48 h in A_p to 72 h in B_p, whilst replacement of medium (C_p) even delayed and decreased the protease production.

Protease produced by the bacterium would hydrolyze protein in the shrimp shells in the fermentation mixture. During the first 12 h incubation, the protein content in the shrimp shells was reduced rapidly from 26% to 13% (±50% protein removed). At the end of observation in A_p; B_p, and C_p 94.42%, 94.51 %, and 94.85 % was removed respectively (Fig 8). Some studies on microbiological demineralization of shrimp shells were also reported. Daum *et al* (2003) showed that fermentation using *Lactobacillus* sp for 48 h could reduce 95% protein of shrimp (*Penaeus* sp) shells. Prameela *et al* (2010) reported that demineralization of *Penaeus monodon* shells for 72 h using *L. plantaraum* NRRL B-14768 removed 76% ash, while Rao and Steven (2005) reported that one step deproteination and demineralization of shrimp shells using *L. plantarum* 541 could removed 83% protein and 88% ash.

Therefore, considering the operational cost, the fermentation design B_m (subsequent batch fermentation) in which 100% medium was replaced with fresh medium after 24 h fermentation, followed by deproteination system A_p (batch fermentation) was suggested. A_p system was the easiest and cheapest compared to the other ones. System A_p needed neither addition of inoculum nor medium.

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