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The Effect of Growth Medium pH towards Trypsin-Like Activity Produced by Lactic Acid Bacteria

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In cases of pancreatic disease, trypsin deficiency often occurs due to reduced expression of trypsin in the pancreas. Patients with pancreatic problem can be treated with a supplement containing digestive enzymes, including trypsin. However, most of the enzymes currently used for the treatment are derived from porcine and bovine sources. On the other hand, lactic acid bacteria are also known to show trypsin-like activity. In the previous work, our group screened 11 lactic acid bacteria isolates, which had previously been proven to show serine protease activity, for trypsin-like activity. The strains were initially grown in MRS (de Mann, Rogosa and Sharpe) medium before being transferred directly to the production medium to produce trypsin. During the previous study, the initial pH of the production medium was set at 6 (the same as the MRS medium pH), which is the optimum pH for the cell growth of lactic acid bacteria. However, most trypsin has an optimum pH of around 8. In this study, we altered the production medium pH to 8 and we harvested the lactic acid bacteria from MRS medium by centrifugation prior to their inoculation to the production medium. Observation of the culture growth and enzyme activity indicated that the new strategy improved the enzyme activity expressed by some strains.

Key words: lactic acid bacteria, trypsin, trypsin-like activity

Pada kasus-kasus gangguan pankreas, defisiensi tripsin seringkali terjadi karena berkurangnya ekspresi tripsin di pankreas. Pasien dengan masalah pankreas dapat ditangani dengan pemberian suplemen enzim pencernaan. Meskipun demikian, sebagian besar enzim yang digunakan untuk pengobatan saat ini didapatkan dari babi atau sapi. Di sisi lain, bakteri asam laktat juga diketahui menunjukkan aktifitas seperti tripsin. Dalam penelitian di grup kami sebelumnya, dilakukan skrining terhadap 11 isolat bakteri asam laktat untuk mendapatkan isolat yang menunjukkan aktifitas seperti tripsin. Isolat-isolat ini sebelumnya telah terbukti memiliki aktivitas protease serin. Isolat-isolat tersebut ditumbuhkan dalam medium MRS (de Mann, Rogosa and Sharpe) sebelum dipindahkan ke medium produksi untuk memproduksi tripsin. Pada penelitian sebelumnya, medium produksi diatur pada pH awal 6 (sama dengan pH medium MRS). Namun demikian, kebanyakan tripsin bekerja pada pH optimum 8. Dalam penelitian kali ini, kami mengubah pH medium produksi menjadi 8 dan melakukan pemanenan sel dengan cara sentrifugasi sel starter dari medium MRS sebelum digunakan untuk menginokulasi medium produksi. Observasi terhadap pertumbuhan kultur dan aktivitas enzim menunjukkan bahwa strategi baru ini meningkatkan aktifitas enzim yang dihasilkan oleh beberapa isolat.

Kata kunci: aktivitas seperti tripsin, bakteri asam laktat, tripsin

Trypsin is a protease enzyme produced by the pancreas and secreted to the duodenum, where it hydrolyses proteins into peptides during the digestion of food. Trypsin is a member of the serine protease family, which digests protein from the carboxyl terminal (C-terminal) of the amino acids lysine (Lys) and arginine (Arg), unless these amino acids are bound to proline (Pro) on their C-terminal (Whitcomb and Lowe 2007).

In cases of pancreatic insufficiency, the capability to produce and/or to transport digestive enzymes, including trypsin, is not sufficient, thus affecting the digestion processes. For this reason, patients with pancreatic insufficiency often show symptoms of malabsorption, malnutrition, vitamin deficiency and weight loss, or in children shown by lack of weight gain (Pezzilli 2009). Pancreatic enzymes have often been used to treat patients with digestion problems caused by a pancreatic defect (Waljee *et al*. 2009). It has been reported that digestive enzyme treatment was proven to increase the fat absorption coefficient of the patients (Waljee *et al*. 2009).

Lactic acid bacteria (LAB) have long been used in food processing. Those which are most commonly used are *Lactococci, Lactobacillus bulgaricus* (Courtin *et al*. 2002; Oberg *et al*. 2002), *L. rhamnosus* (Haq *et al*. 2006), *L. casei* (Piuri *et al*. 2003), *L. paracasei* (Bintsis *et al*. 2003; Haq and Mukhtar 2006), *L. helveticus* (Oberg *et al*. 2002), *L. delbrueckii* (Oberg *et al*. 2002), *L. brevis*, *L. cellobiosus*, *L. fermentum*, and *L. plantarum* (Haq and Mukhtar 2006). In addition,

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several bacteria from the genus *Lactobacillus* are known to have proteolytic activity (Caplice *et al*. 1999; Haq and Mukhtar 2006).

Proteolysis of milk proteins by LAB plays an important role in providing peptides and amino acids to cover the demand of the bacteria and previously several LAB known to have trypsin-like activity have been isolated (Wulansari *et al*. 2010). The screening was performed on isolates previously known to show serine protease activity (Wulansari and Wahyuntari 2010). The strains used to produce the enzyme were cultured in two stages, the growth stage and the production stage. During the growth stage, the strains were cultured in MRS (de Mann, Rogosa and Sharpe) medium, whilst during the production stage, the strains were cultured in the production medium (Glucose 0.5%, Yeast Extract 0.1%, Skim Milk 1%, pH 6) (Wulansari and Wahyuntari 2010). In this project, a medium with pH 8 was used and prior to inoculation to the production medium, cells were harvested from the growth medium by means of centrifugation.

MATERIALS AND METHODS

Strains and Media. The strains used in this study (listed in Table 1) were from the collection of Centre for Bioindustrial Technology, BPPT, Indonesia.

The medium used for growth of the lactic acid bacteria was the MRS medium (Oxoid CM0359), which was autoclaved at 121 °C for 15 min.

The media used for trypsin production contains glucose 0.3%; yeast extract 1%; skim milk 2%; pH 6 or 8, and was autoclaved at 121 °C for 15 min.

Culture. Strains from 40 µL glycerol stocks were regenerated in 4 mL MRS medium broth incubated at 37 °C, shaking at 50 rpm for 18 hours. The culture was then used to inoculate fresh MRS medium broth (1% inoculum) which was incubated at 37 °C with shaking at 50 rpm for 4-6 hours. This starter was then used to inoculate the trypsin production medium. Two kind of production media and methods were used and compared (Fig 1).

Method 1. Starter was used to inoculate production

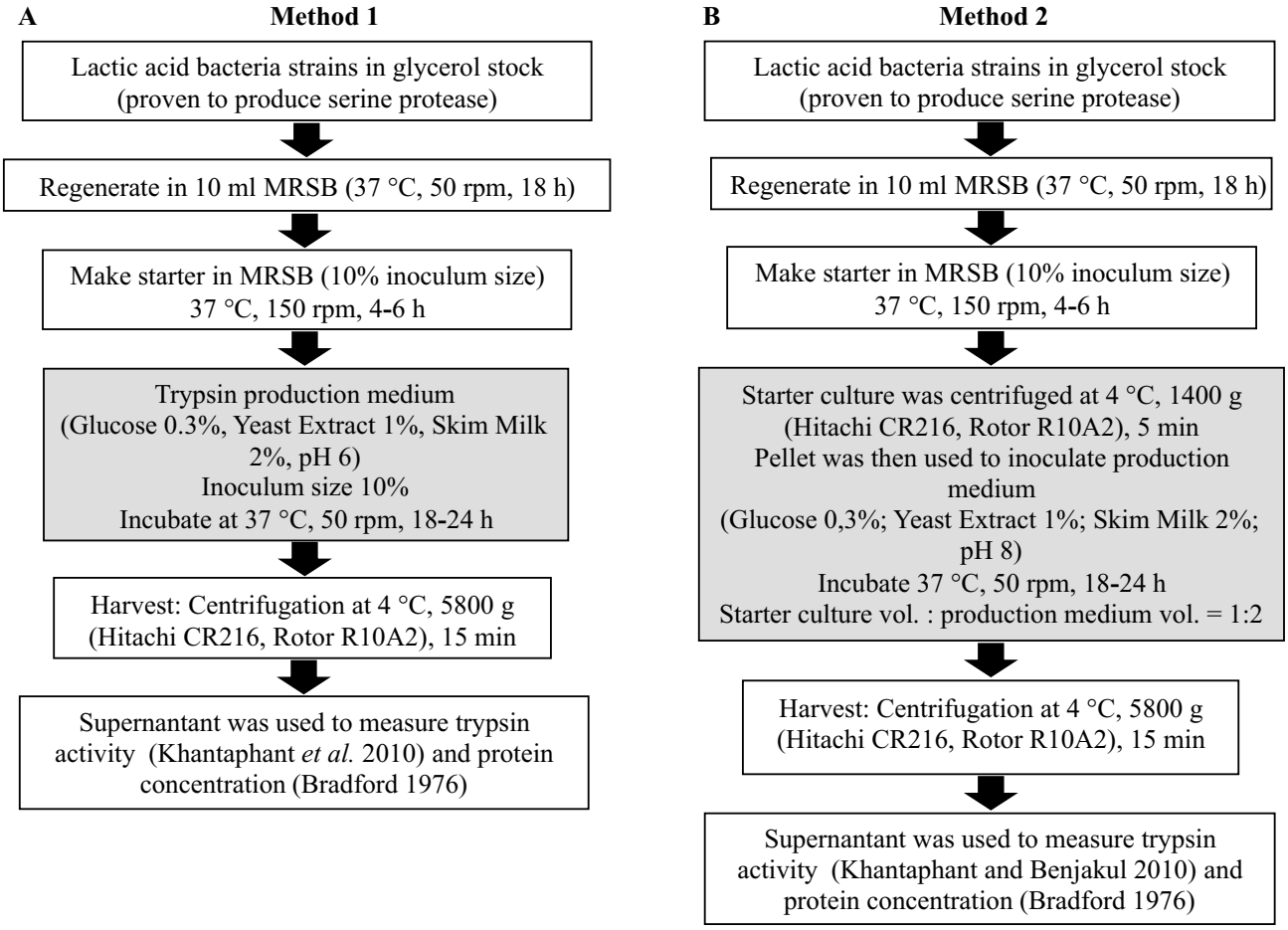

Fig 1 The methods used in the cultivation of lactic acid bacteria to produce trypsin-like protease. Squares shaded in grey show the two different methods used. A) Method 1: production medium pH 6, starter culture transferred directly to the production medium as described in previous publication (Wulansari and Wahyuntari 2010); B) Method 2: production medium pH 8, starter cultures were centrifuged prior to transferred to the production medium.

medium (glucose 0.3%, yeast extract 1%, skim milk 2%, pH 6) with inoculum size 10%. The culture was then incubated at 37 °C with stirring at 50 rpm for 18-24 h.

Method 2. Starter culture was centrifuged at 4 °C, 1400 g (Hitachi CR216, Rotor R10A2) for 5 min and the pellet was then used to inoculate the production medium (glucose 0,3%, yeast extract 1%, skim milk 2%, pH 8). The production culture was then incubated at 37 °C, with 50 rpm stirring for 18-24 h. The ratio of starter culture volume : production medium volume is 1:2.

Each method was performed at least twice on each strain studied, thus the error bars, as shown in the figures, represent the errors obtain from these repeated experiments. The supernatants were separataed from cells by centrifugation (4 °C, 5800 g (Hitachi CR216, Rotor R10A2), 15 min) and the enzyme activities in the supernatants were measured.

Trypsin Activity Assay. Trypsin activity was measured using α-N-benzoyl-DL-arginine-ρ-nitroanilide (BAPNA) as substrate, using a modified Khantaphant and Benjakul method (Khantaphant and Benjakul 2010). For the assay, 50 µL enzyme was mixed with 50 µL MilliQ water and 250 µL reaction buffer, and left for 5 min at 37 °C . The reaction buffer used was either TrisHCl 0.05M pH 8 or citrate buffer 0.05M pH 6. The pH of the reaction buffer used for the assay depends on the medium pH used for the enzyme production. The reaction was initiated by addition of 50 µL BAPNA (Sigma B4875) 2 mg mL^{-1} and was allowed to continue for 20 min at 37 °C. To stop the reaction, 50 µL 30% acetic acid was added to the reaction mixture. Production of ρ-nitroaniline was measured by measuring the absorbance of the reaction mixture at 410 nm. A blank reaction was conducted in the same manner, except that the sample was added after addition of the acetic acid.

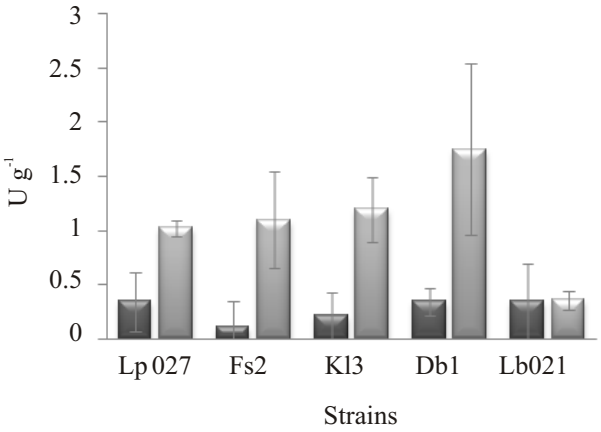

Fig 2 Specific trypsin like activities produced by the isolates. Isolates Lp027, Fs2, Kl 3, and Db1 showed differences in activities when produced using different conditions, and thus studied further. Isolate Lb021 expressed similar activity whether produced using Method 1 or 2. ■ Trypsin-like activity when produced using Method 1; ■ Trypsin-like activity when produced using Method 2.

Trypsin activity was calculated as follows:

$$\text{Trypsin activity (unit/ml)} = \frac{(A\text{-}A_0) \text{ x mixture volume (ml) x } 1000}{8800 \text{ x reaction time (min) x } 0.2}$$

where 8800 ($cm^{-1}M^{-1}$) was the extinction coefficient of ρ-nitroaniline; A and A_0 were the sample and blank absorbances at λ 410, respectively.

One unit of enzyme activity was defined as the activity that causes the release of 1 nmol ρ-nitroaniline per minute.

Trypsin Inhibition Assay. The trypsin inhibition assay was performed using soybean trypsin inhibitor (SBTI) with activity 1000 U mg^{-1} (Sigma, cat. Number 93620). The assay was performed by dissolving 0.5, 1, 1.5, 2, 2.5 mg SBTI in 1 mL enzyme and then left to incubate at room temperature for 15 min. Then, the trypsin activity of the mixture was assayed. The percent inhibition was calculated by the following formula :

$$\frac{\text{Activity}_{\text{Not-Inhibited}} - \text{Activity}_{\text{Inhibited}}}{\text{Activity}_{\text{Not-Inhibited}}} \text{ x } 100\%$$

pH and Temperature Profile. The assay to determine the pH and temperature pofile was performed as described previously by Khantaphant and Benjakul (2010). The optimum temperature as determined by the temperature profile was then used for the measurement to determine the pH profile.

Trypsin Stability Assay. Stability assays were performed to check trypsin stability in the range of pH and temperature values tested. The assays were performed based on Khataphant and Benjakul (2010) method . Trypsin stability was determined by measuring the remaining activity after the enzyme was treated at various pH and temperature values. For thermal stability, the enzyme was incubated at temperatures 24, 35, 45, 50, 55, 60, 65, and 70 °C for 30 min before transferred to ice. Then, the residual activity was measured. For pH stability, trypsin was mixed with an equal volume of the appropriate buffer, and left for 30 min at room temperature. The buffer used was either 0.05M acetic acid-sodium acetate buffer for pH 4-6 or 0.05M Tris HCl buffer for pH 7-9 (Khantaphant and Benjakul 2010). The activity was then assayed at the optimal pH and temperature using (Benzoyl arginine ρ-nitroanilide) BAPNA as the substrate.

RESULTS

Changing the pH of the Production Medium and Inoculation Method Changed the pH of the Culture. The different methods used for trypsin production seemed to affect the medium pH at the time of inoculation and at the time of harvest. Before used for inoculation, the pH of starter cultures range between 4.87-5.74. The initial pH of the production cultures inoculated using Method 1 ranged between 4.95-5.65 and went down to between 3.15-3.85 at the time of harvest. However, when the method was altered, the pH at time of inoculation increased to between 7.07-7.93 and retained around the level of 6.24-7.15 at the time of harvest. Harvesting was performed when trypsin-like activity reached maximum levels during culture growth (Table 1), as previously optimized. Trypsin-like activity of the cultures were assayed and the results showed that when the bacteria were cultured using Method 2, four strains, namely Lp027, Db1, Fs2, and Kl3, showed a considerable increase of trypsin like-activity. One strain, however, Lb021, showed exactly the same activity whether the enzyme was produced using Method 1 or 2. The rest of the strains tested showed no considerable trypsin-like activity, whether cultured using Method 1 or 2.

Table 1 Strains used in the study, their origins (Susanti et al. 2007) and harvest time

Strains	Origin	Harvest time (hours after inoculation)
Lc 262	Fermented glutinous rice	18
Lp 027	Growol*	18
Fs 2	Infant faeces	18
Kj 1	Cheese	18
Sk 3	Horse milk	18
Kl 3	Coconut water	21
Db 1	Dadih**	21
T 3	Earth	21
Lb 021	Fermented glutinous rice	24
Kj 3	Cheese	24
Kl 2	Coconut water	24

*Growol is an Indonesian traditional food made of cassava
**Dadih is an Indonesian traditional drink made of fermented buffalo milk. It is similar to yoghurt in taste and appearance

Changing the pH of the Production Medium and Inoculation Method Affected the Growth Rate and the Specific Trypsin-Like Activity. The four strains that showed considerable increase of activity were then cultured using two different methods (Method 1 and 2) as described earlier. The culture pH, number of cells and trypsin-like activity were observed every six hours and the values were plotted against time (Fig 3 and 4). The figures clearly show that the change of method used for culturing seemed to have affected not only the culture pH, but also the maximum trypsin-like activity shown by the cultures, although the maximum cells numbers seemed unchanged. Since Method 2 seemed to produce higher trypsin-like activity, this method was then selected as the better of the two methods. Therefore, the characteristics of enzymes produced using method 2 were further explored.

Trypsin-Like Activities Shown by the Four Strains Were Inhibited by Soybean Trypsin Inhibitor (SBTI). Soybean trypsin inhibition assays were performed on enzymes produced using Method 2 to prove that the activity was really caused by trypsin. When treated with SBTI, the trypsin like-activity of the four strains seemed to be considerably inhibited (Fig 5). The percent inhibition also increased, although not to the same extent, with the increase of SBTI concentration.

pH and Temperature Profiles and Stabilities. The pH and temperature profiles showed that for all strains, the optimum pH was observed around pH 8 and the optimum temperature was observed around 36-38 °C (Fig 6). The trypsin-like activity seemed to be retained above 60% when enzymes were heated at temperature range 37-38 °C (Fig 7). The pH stability assay shows somehow more divergen result. Although the trypsin-like activity expresed by Fig 2-K13 seemed to be most unaffected when incubated at pH 8 for 30 min prior to activity measurement, this pH value considerably reduced the activity expressed by strains Lp027 and Db01. On the other hand, the trypsin-like activity expressed by Lp027 seemed to be unaffected after incubation at pH 7.5, whereas, Db1 retained the activity the best at pH 7.0.

DISCUSSION

Previous publication described the selection of strains producing trypsin-like activity from LAB collections known to express serine protease activity (Wulansari and Wahyuntari 2010). All the trypsin-like activites observed in the previous publication and in the current study were based on extracellular measurements. As described by Liu *et al.* (2010), there are three major components of LAB proteolytic systems, which are the cell wall bound proteinases initiating the degradation of the extracellular caseins, the peptide transporters taking up the peptides into the

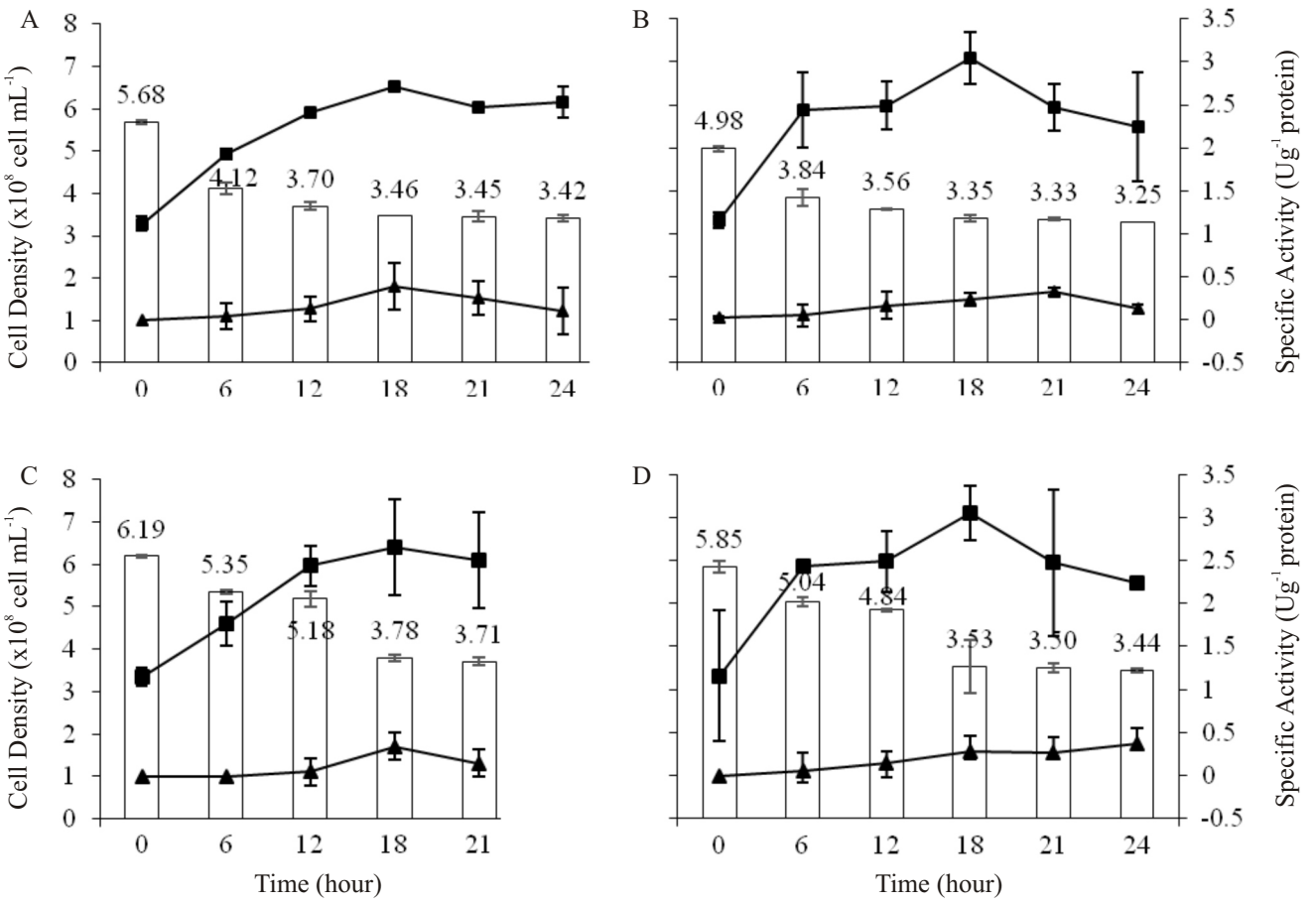

Fig 3 Changes of pH, cell number and trypsin specific activity of strains (A) Lp027, (B) Db1, (C) Fs2, and (D) Kl3, cultured using Method 1, the culture pH is represented in bar and the value for each time point is written above the bar, ■ cell numbers (x10^8 cells mL^{-1}), ▲ specific activity (U g^{-1} protein).

cells, where they would be further digested by various intracellular peptidases (Liu *et al.* 2010). Searching the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein) using keywords trypsin and lactic acid bacteria several proteins from LAB reported to show trypsin-like activity were identified. This, obviously, is relevant to the observed trypsin like activity observed in some of the strains screeed earlier (Wulansari and Wahyuntari 2010).

In the previous work, production medium with pH 6 was used (Wulansari and Wahyuntari 2010). This pH value was used because the cultures were initially grown in MRS medium (pH 6) prior to inoculation to the production medium. In addition, pH around 6 was considered the optimum pH required for the growth of lactic acid bacteria (Hutkins *et al.* 1993). The production medium was inoculated by directly transferring 10% inoculum previously grown in MRS edium (Method 1), without separating the cells from the growth medium. Using this method, the production medium pH immediately dropped to between 4.98-6.19 (Fig 3). The pH further decreased until it reached between 3.25-3.71 at the end of the fermentation (Fig 3), which is probably due to the secretion of lactic acid by the bacteria. This condition is, of course, not good for the survival of the bacteria, since they are very prone to cell damage when the final medium pH goes below pH 5.0 (Hutkins and Nannen 1993). The low pH phenomenon was observed on cultures grown using Method 1. The low pH of the cultures could have considerably reduced cell viability, since lactic acid bacteria in general grow at pH range 4.5-7.0 (Hutkins and Nannen 1993).

Regardless of the cells viability, low pH might also have affected the trypsin-like activity. As is widely known, trypsin works optimally at pH around 7.5-8.5 (Koutsopoulos *et al.* 2007). At pH lower or higher than this range the activity would most likely be reduced, thus the lower pH might have considerably reduced the trypsin-like activity expressed by the strains tested.

Harvesting the cells by centrifugation was aimed at isolating cells from the acidic environment of the growth medium (see Table 2 for pH of the starter culture). By harvesting the cells prior to their use for inoculation, the amount of growth medium, *i.e.* acidic medium, being carried over to the production medium

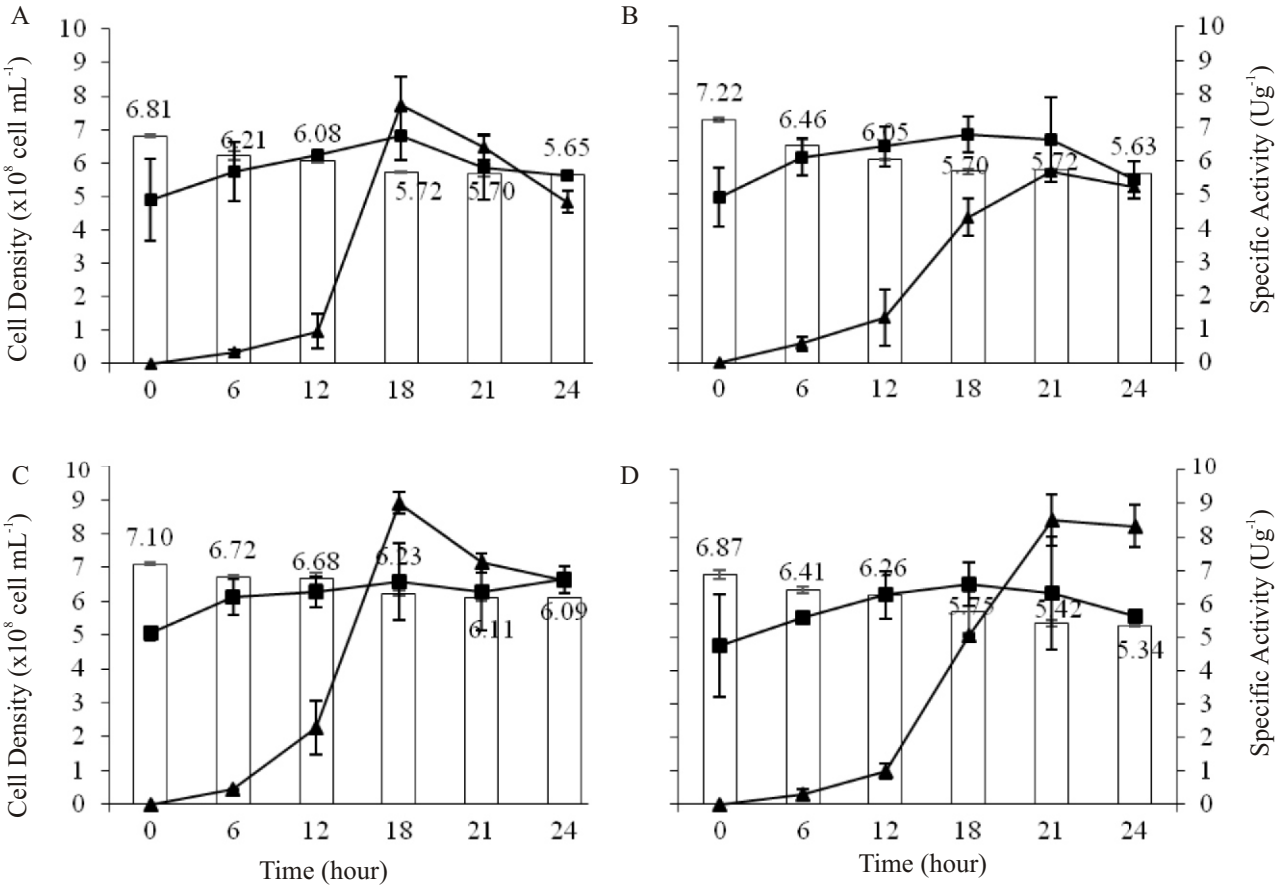

Fig 4 Changes of pH, cell number and trypsin specific activity of strains (A) Lp027, (B) Db1, (C) Fs2, and (D) Kl3, cultured using Method 2, the culture pH is represented in bar and the value for each time point is written above the bar, ■ cell numbers (x108 cells/ml), ▲ specific activity (U g^{-1} protein).

was minimised. Thus, the sudden decrease of production medium pH immediately after inoculation as demonstrated when Method 1 was used (pH after inoculation 4.98-6.15) was prevented. Since during their growth lactic acid bacteria produce lactic acid, the end pH of the production medium was 3.25-3.71 when Method 1 was used. On the other hand, when Method 2 was used, the production medium pH was retained at around 7 at the beginning and around 6 at the end of the fermentation. Although the extent of pH decrease in Method 1 and 2 was more or less equal (i.e. around 1-2 pH Unit), the end pH in Method 2 remained above 6. The relatively more stable pH seemed to have been able to retain the trypsin like-activities expressed by the culture when produced using Method 2.

There could be two underlying reasons why the trypsin like-activities were higher in Method 2. It was possible that at the higher pH, more bacteria could survive and so more enzymes were produced, or that at higher pH the enzyme was more stable compared to at lower pH, that the typsin like-activities were higher. Looking at the numbers of cells at the time of harvest

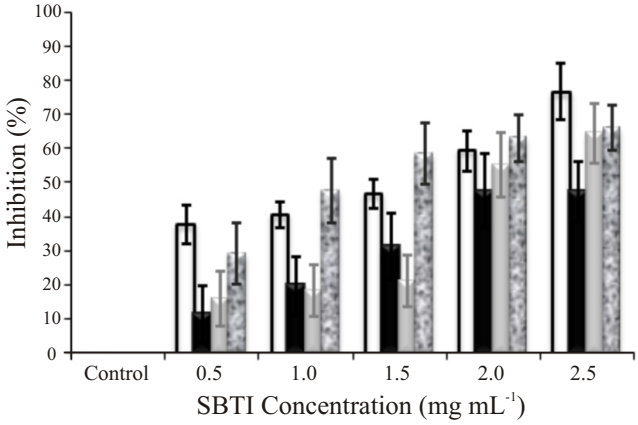

Fig 5 Percent inhibition by soybean trypsin inhibitor (SBTI) of enzyme activity produced using Method 2, Lp027, Db1, Fs2, Kl3.

(i.e. 18 h), it seems that the difference in cell numbers was unlikely to be the reason, because at 18 h the cell densities of all cultures, whether grown at pH 6 or 8, were approximately the same at around 7 x 10^8 (Fig 3 and 4). To further prove that the enzyme activities observed were really produced by trypsin, trypsin inhibition assays using soy bean trypsin inhibitor

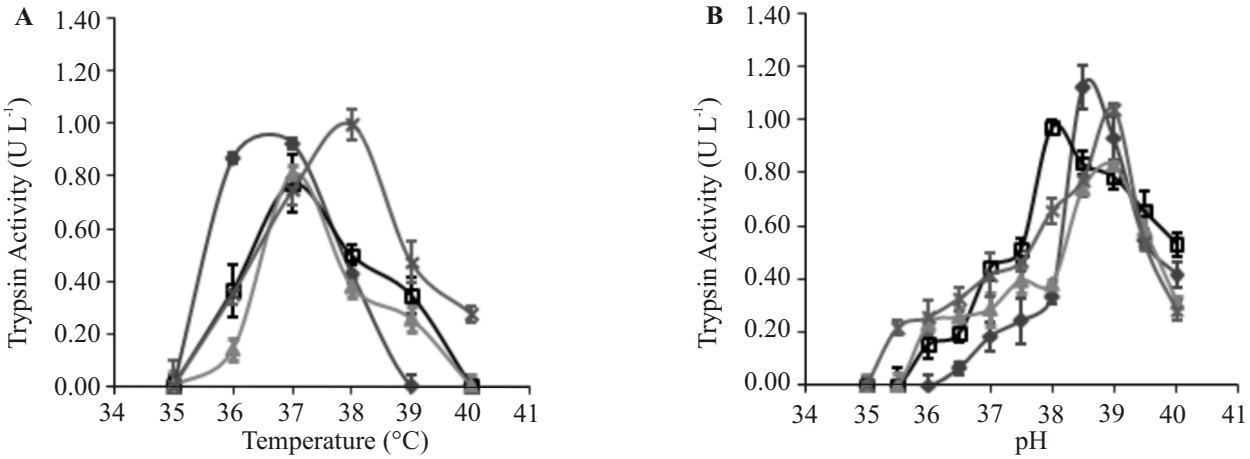

Fig 6 Temperature(A) and pH (B) profile of the trypsin like activity of the enzymes produced using Method 2. The assays were performed as described previously by Khantaphant and Benjakul (2010). The optimum temperature as determined by the temperature profile was then used for the measurement to determine the pH profile. ◆Lp027, ⊟Db1, ▲Fs2, ✕Kl3.

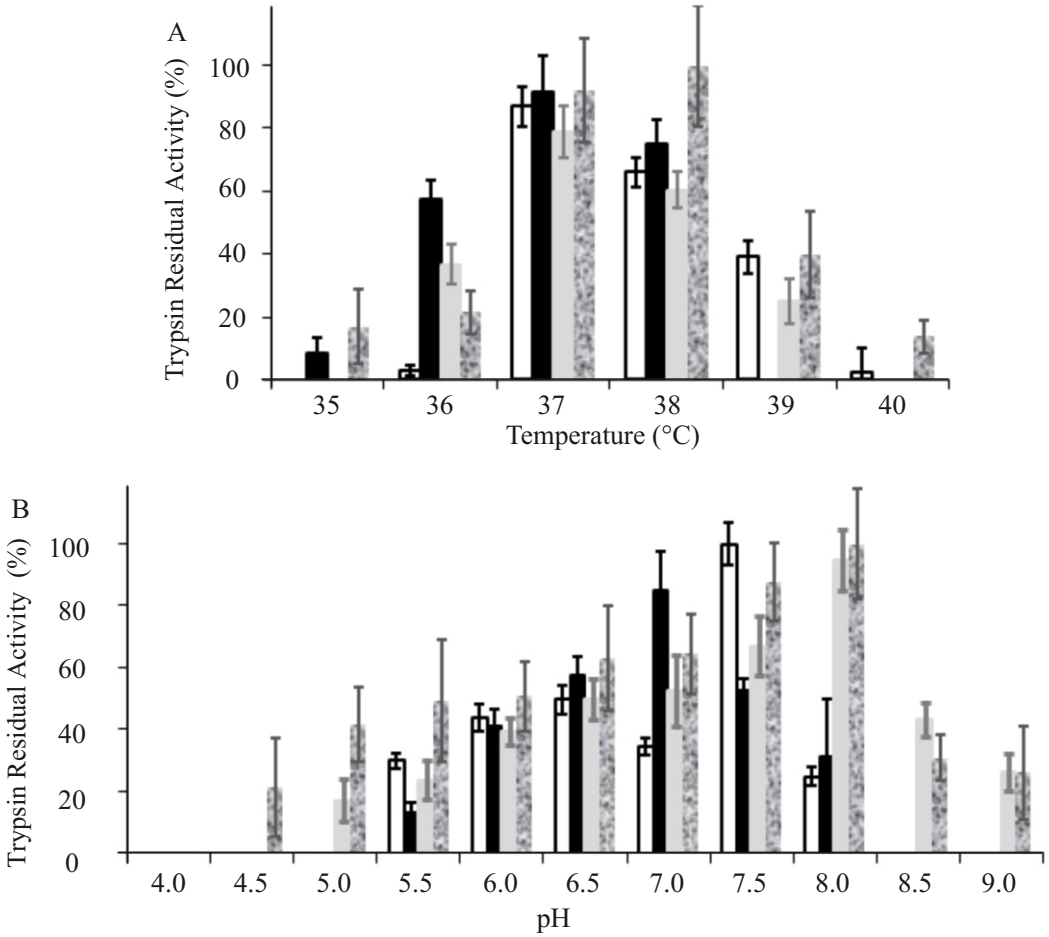

Fig 7 Residual activity of trypsin produced by Method 2, after treatment with various temperature (A) and pH (B). For thermal stability, the enzyme was incubated at temperatures 25, 35, 45, 50, 55, 60, 65, and 70 °C for 30 min before transferred to ice. Then, the residual activity was measured. For pH stability, trypsin was mixed with an equal volume of the appropriate buffer (either 0.05M acetic acid - sodium acetate buffer for pH 4 - 6 or 0.05M Tris HCl buffer for pH 7 - 9 (Khantaphant and Benjakul 2010)), and left for 30 min at room temperature. The activity was then assayed at the optimal pH and temperature (as determined by temperature and pH profile analyses) using BAPNA as the substrate. □Lp027, ■Db1, ▒Fs2, ▒Kl3.

(SBTI), were preformed (Fig 5). The assays indicated that the activities expressed by all strains were inhibited by SBTI, although to a different extent with different cultures. The amount of SBTI required for the inhibition, however, was higher in comparison to the amount required to inhibit trypsin from other sources

(Struthers and MacDonald 1983), including bacteria (Takada *et al.* 2000; Hansen *et al.* 2005).

The trypsin like-activities of the strains were stable at range between 36-38 °C and the effect of temperature treatment was least when the enzymes was treated at 37 °C for 30 min prior to measurement. The pH stability studies showed that the trypsin like-activities were still detectable after treatment at pH range 5.5-8 for 30 min prior to activity measurement (Fig 7). The pH and temperature profiles also showed more or less the same patterns, with optimum temperature around 36-38 °C, optimum pH aroun 7-8. This is relevant to the general knowledge that trypsin has an optimum temperature of 37 °C and an optimum pH between 7.5-8.5 (Koutsopoulos *et al*. 2007). Considering the results of inhibition experiments, as well as the patterns of temperature and pH profile and stability, shows that the activities observed were really expressed by trypsin from the lactic acid bacteria. N-terminal sequencing would be necessary to be entirely sure the protein is pure enzyme.

In conclusion, it is clear that the methods used for inoculation of production medium, in addition to the production medium pH, affect the trypsin-like activity. As clearly and thoroughly presented above, using production medium with pH 8 and separation of cells from growth medium prior to inoculation to the production medium, increased the trypsin-like activity produced. This is a better method for trypsin production from lactic acid bacteria.

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