CHAPTER 4

RESULTS AND DISCUSSION

4.1 Screening of protease producing strains

4.1.1 Protease production

In order to improve the productive performance that target to protein in soybean meal (SBM), protease production of 19 isolates of *Bacillus* sp. was primarily evaluated based on hydrolysis of soy protein isolate at pH 6.8 and 39.5°C. Among 19 *Bacillus* sp. isolates from either silage or fermented foods, *Bacillus* sp. FAS014 showed the highest specific activity of protease, followed by *Bacillus* sp. FAS0018, *Bacillus* sp. FAS001, *Bacillus* sp. FAS019, and *Bacillus* sp. FAS003, respectively (Figure 4.1). *Bacillus* sp. produce a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. *Bacillus* sp. are of the most widely used bacteria for the production of enzyme and specialty chemicals (Samal *et al*., 1990).

4.1.2 Resistance to pH and heat treatment

Due to the acid condition in the animal's digestive tract, further screening of selected strains of *Bacillus* sp. was primarily based on its resistance to low pH. Figure 4.2 shows the remaining protease activity of crude protease from selected *Bacillus* sp. after exposure to low pH. Substantial decrease in protease activity was observed in all selected strains of *Bacillus* sp. tested. After incubation for 60 min at pH 3.0, the residual activity of protease was approximately 16.6%, 9.3% and 4.8% for the protease from *Bacillus* sp. FAS001, *Bacillus* sp. FAS0014 and *Bacillus* sp. FAS018, respectively.

Figure 4.3 shows the remaining protease activity after exposure to heat treatment at 55-85°C for 10 min. Proteases of selected strains were remarkably stable at 55-60°C with a residual activity greater than 80% compared to control (without heat treatment). Increase in temperature resulted in the significant loss of activity. At 65°C, the protease from *Bacillus sp*.FAS001 was the most stable. At incubation higher than 70°C, the residual protease activity of selected strains was less than 10% compared to control.

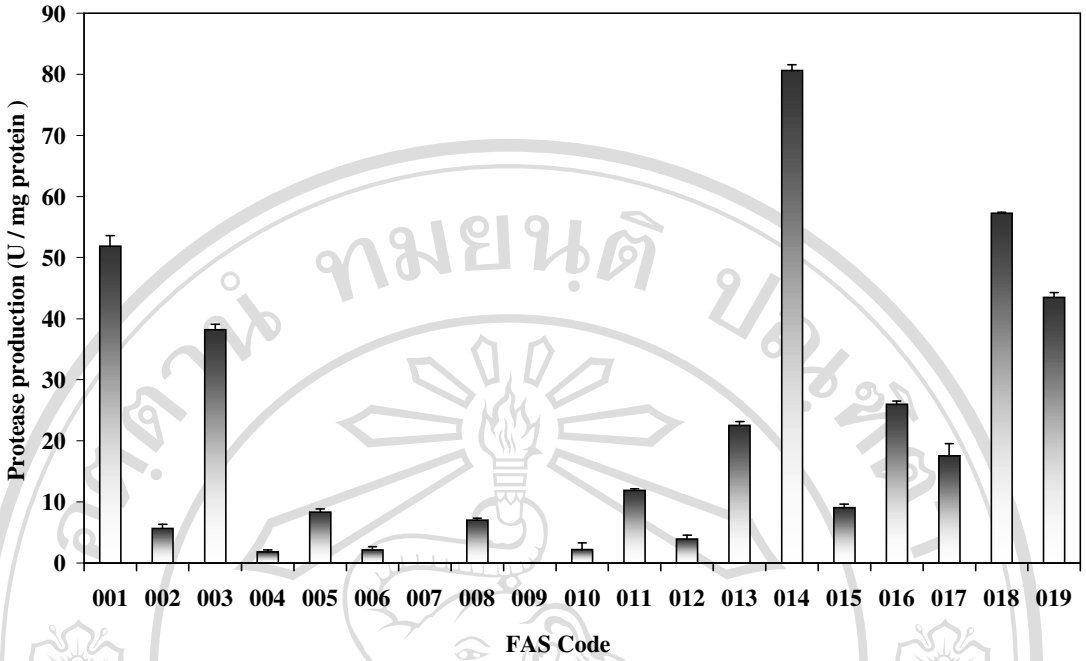

Figure 4.1 Protease production of *Bacillus* sp. FAS 001-019; Bars indicate standard deviations from 3 determinations. Activity was assayed at pH 6.8, 39.5°C for 10 min by using soy protein isolate as substrate.

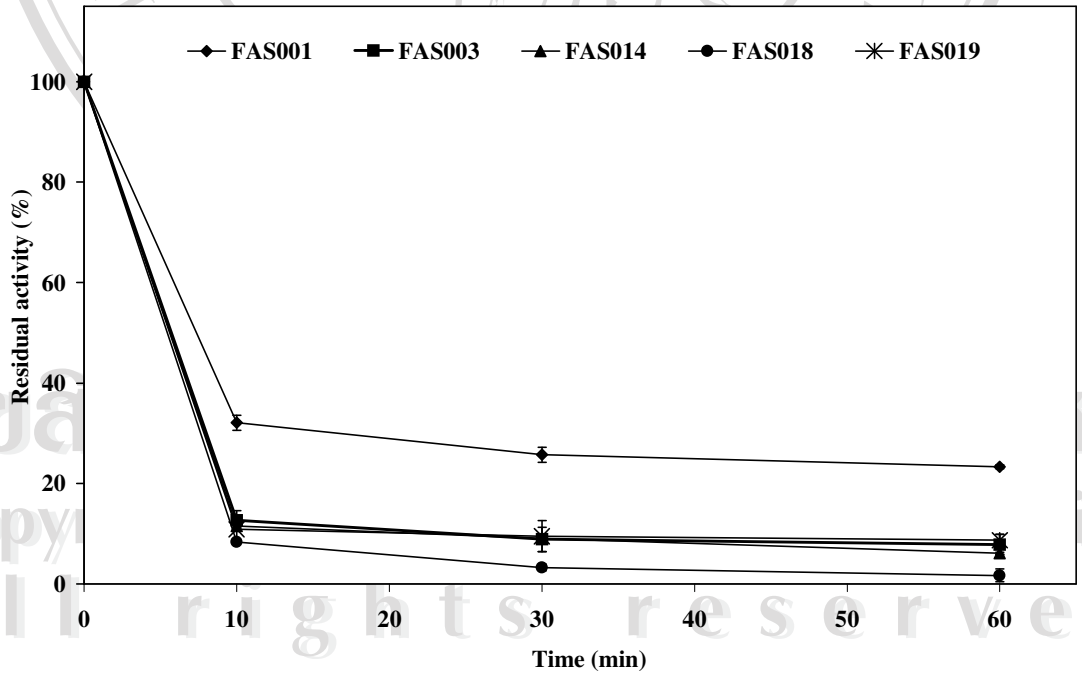

Figure 4.2 Stability of protease at pH 3.0; Bars indicate standard deviations from 3 determinations. Activity was assayed at pH 6.8, 39.5°C for 10 min by using soy protein isolate as substrate.

The thermal stability at 75°C of protease from selected *Bacillus* sp. is illustrated in Figure 4.4. The protease activity of all selected strains decreased with the increasing exposure time at 75°C. After incubation for 2 min, the protease from *Bacillus* sp. FAS001 was the most stable, among all selected strains tested. With the exception of protease from *Bacillus* sp. FAS003, the residual activity decreased to 5% after incubated at 75°C for 5 min. The temperatures which feeds are exposed during the pelleting process can range from 60 to 90°C under normal conditions. These temperatures and pressures can therefore lead to loss of feed-borne and added enzyme activity (Rexen, 1981). Therefore, it was likely that protease from *Bacillus* sp. FAS001 could be used under pelleting conditions (75°C, 30-60 s) with little loss of protease activity.

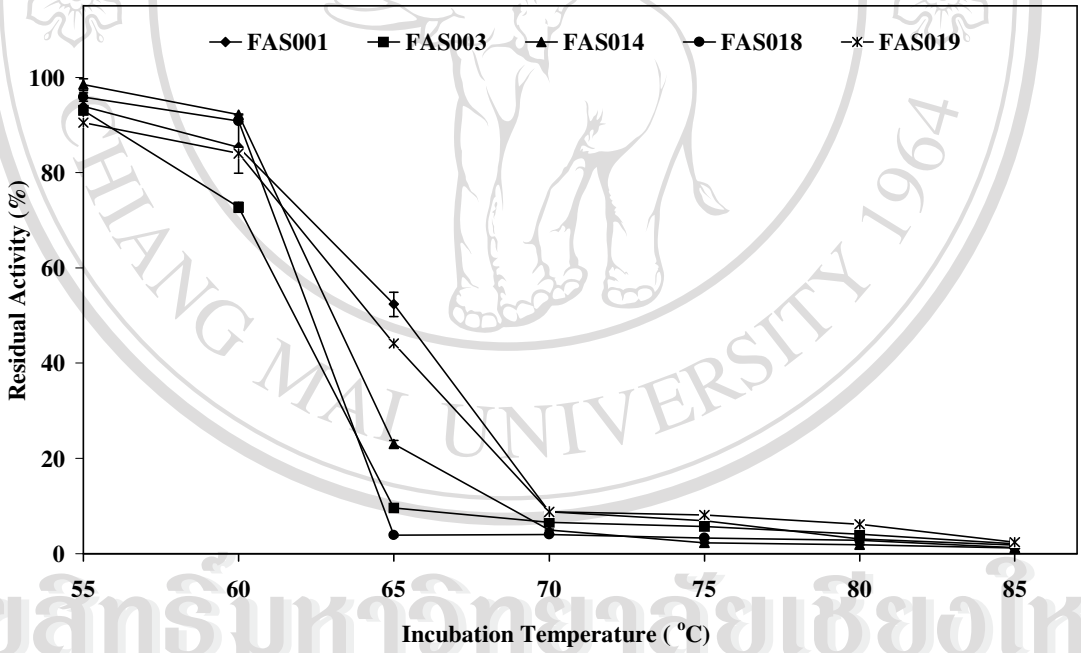

Figure 4.3 Thermal stability of protease after incubation at 55-85°C; Bars indicate standard deviations from 3 determinations. Activity was assayed at pH 6.8, 39.5°C for 10 min by using soy protein isolate as substrate.

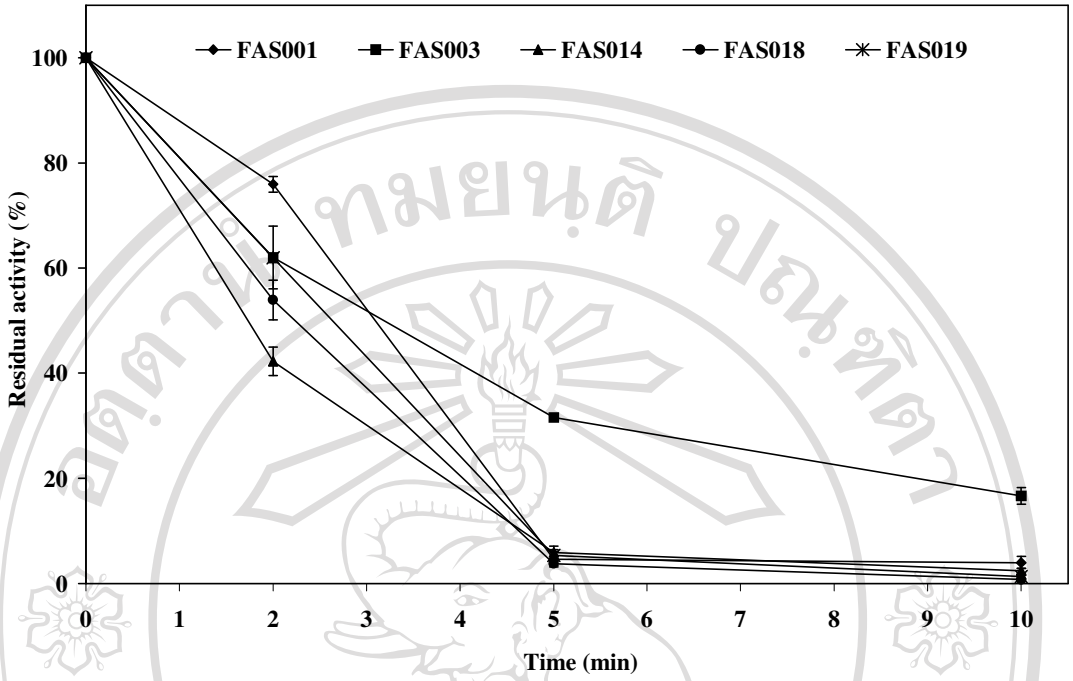

Figure 4.4 Thermal stability of protease after incubation at 75°C; Bars indicate standard deviations from 3 determinations. Activity was assayed at pH 6.8, 39.5°C for 10 min by using soy protein isolate as substrate.

4.1.3 Resistance to protease inhibitors

The effect of various types of protease inhibitor on protease activity is shown in Figure 4.5. Varying levels of inhibitor were observed depending on strains and type of inhibitors. The results suggested the differences in molecular and catalytic properties of major protease in crude protease preparation. Maximum inhibition by EDTA and EGTA (>60%) indicated that crude protease from *Bacillus* sp. were predominantly metallo-protease. Different from EDTA, higher inhibition by EGTA which bind specifically to Ca^{2+} ion suggested that FAS018 required Ca^{2+} ions for enzymatic activity. Varying level of inhibition (30-50%) by PMSF. suggested the presence of serine protease as the second most predominant protease. *Bacillus* sp. possess the ability to excrete several hydrolytic enzymes into the cultivation medium. Almost all species of *Bacillus* sp. are able to secrete proteases. The maximum rate of exogenous synthesis is usually observed in the late exponential or early stationary phase of growth at which time that the cells begin preparation for sporulation (Debabov, 1982).

Two prominent proteases was produced during the onset of sporulation are the alkaline proteases (Uehara et al., 1979; Tsuru et al., 1966). The combined activities of these two enzymes account for 96-98% of the total protease activity present in the culture supernatant of wild-type sporulating cells (Kawamura and Doi, 1984).

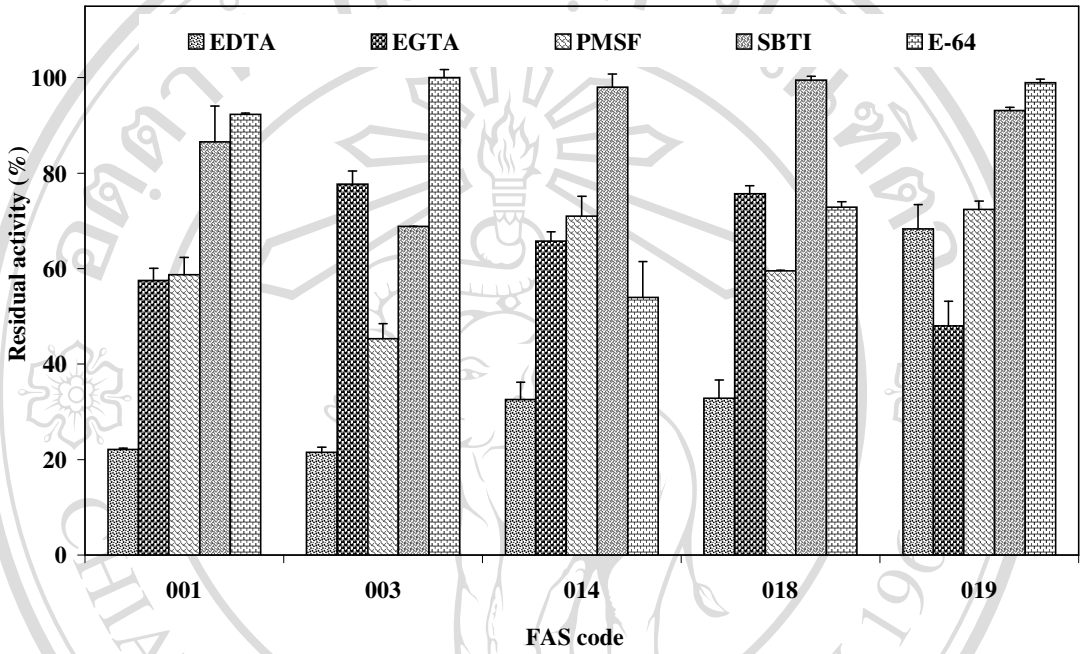

Figure 4.5 Effect of protease inhibitors on crude protease activity; Bars indicate standard deviations from 3 determinations. Activity was assayed at pH 6.8, 39.5°C for 10 min by using soy protein isolate as substrate.

From screening, *Bacillus sp*. FAS014 showed the highest specific activity of protease followed by *Bacillus* sp. FAS0018 and *Bacillus* sp. FAS001, respectively. However, protease from *Bacillus* sp. FAS001 was found to be the most stable either at pH 3.0 or under heat treatment. With about 80% of the activity retained after incubation at the pelleting temperature and high resistance to soybean trypsin inhibitor, *Bacillus* sp. FAS001 was the most favorable strain for protease production.

4.2 Optimization of crude protease production

4.2.1 Effect of SBM concentration

Soybean meal (SBM) was used as the sole carbon and nitrogen sources in the basal cultured medium. In order to optimize concentration of SBM, minimal synthetic medium containing different SBM concentrations ranging from 0 to 8% (w/v) was inoculated with 0.2 ml of the 15-h seed and was aerobically incubated at 37°C for 24 h on a rotary shaker (150 rpm). Figure 4.6 shows the effect of SBM concentration on growth and protease production. Without SBM, no growth and protease production was observed. Maximum growth and protease production were observed at 2% (w/v) SBM. Increase in SBM concentration above 2% resulted in lower protease production without any effects on growth. Therefore, medium containing 2% (w/v) was optimal for protease production. SBM was recognized as a potentially useful and cost-effective medium ingredient, because it is largely produced as a by-product during oil extraction (Joo *et al*., 2002). SBM was used as the sole source of carbon and nitrogen along with mineral solution for protease production of *Bacillus alcalophilus.* (Kanekar *et al*., 2002). The increasing of substrate concentration also limited the oxygen tension of the medium resulting in decreased the growth and protease duction.

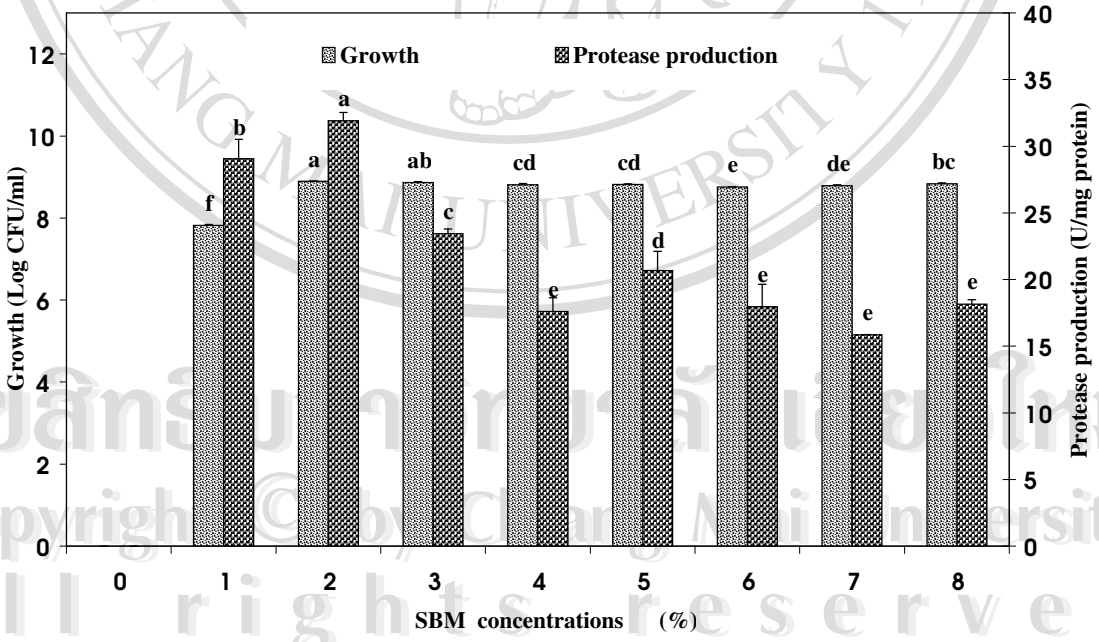

Figure 4.6 Effect of SBM concentrations on growth and protease production of *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations. Different letters (a,b,c,d,e,f) within the same parameter indicate significant differences (P<0.05).

4.2.2 Effect of initial pH

A 125-ml Erlenmeyer flask containing 20 ml of optimized medium from 4.2.1. adjusted pH 4.0, 5.0, 6.0, 7.0 and 8.0 was inoculated with 2% (v/v) of the 15-h seed and incubated at 37°C for 24 h on a rotary shaker (150 rpm). The effect of initial pH on growth and protease production is shown in Figure 4.7. Maximum growth and protease production was observed at pH 5.0 ($P<0.05$). The growth and protease production decreased with increasing initial pH. It was noted that at initial pH 8.0, no growth and protease production were observed. The culture pH strongly affects many enzymatic processes and transport of various components across the cell membrane (Moon and Parulekar, 1991). In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds, the medium turned more alkaline during protease production (Moon and Parulekar, 1993). As a consequence, higher cultivation pH than optimum pH may lead to a decreased the rates of enzymatic reaction.

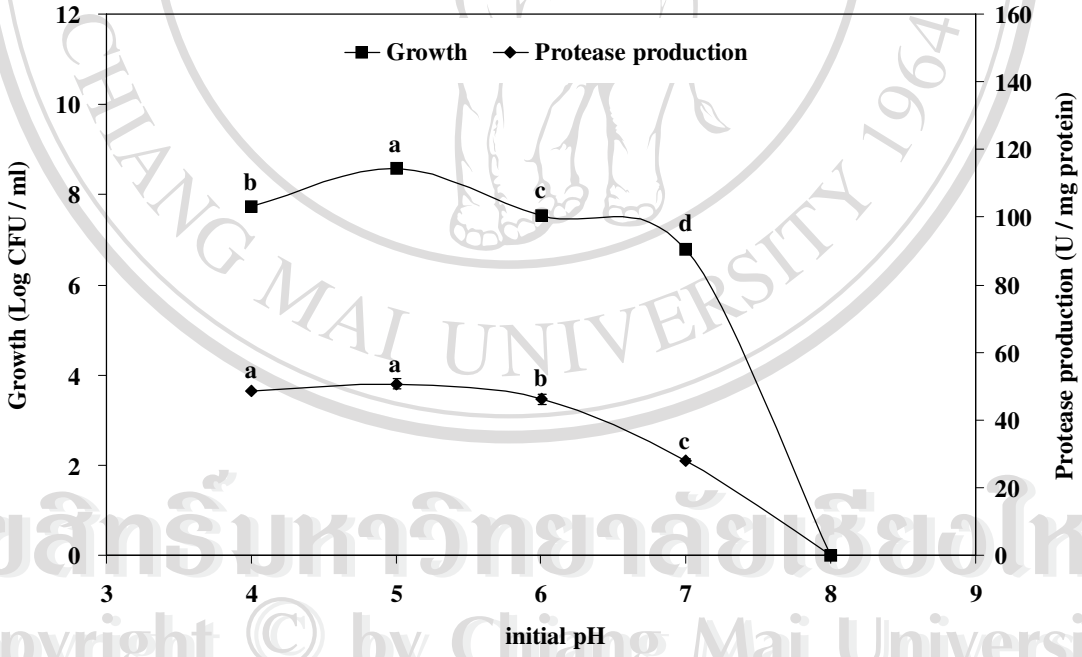

Figure 4.7 Effect of initial pH on growth and protease production of *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations. Different letters (a,b,c,d) within the same parameter indicate significant differences ($P<0.05$).

4.2.3 Effect of cultivation temperature

A 125-ml Erlenmeyer flask containing 20 ml of optimized medium from 4.2.2. was inoculated with 0.2 ml of the 15-h seed and incubated at various temperatures ranging from 25, 30, 35, 37 or 40°C for 24 h on a rotary shaker (150 rpm). Effect of cultivation temperature on growth and protease production is shown in Figure 4.8. No significant effect of temperature on growth of *Bacillus* sp. FAS001 was observed. However, the protease production increased with the increasing cultivation temperature and the maximum protease production was observed at 37-40°C. This result showed *Bacillus* sp. FAS001 had broad range temperature for growth and protease production. The optimal growth temperature of Mesophilic *Bacillus* sp. is generally observed at 30-45°C. The mechanism of temperature control of enzyme production is not well understood (Chaloupka, 1985). However, Frankena *et al*. (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake.

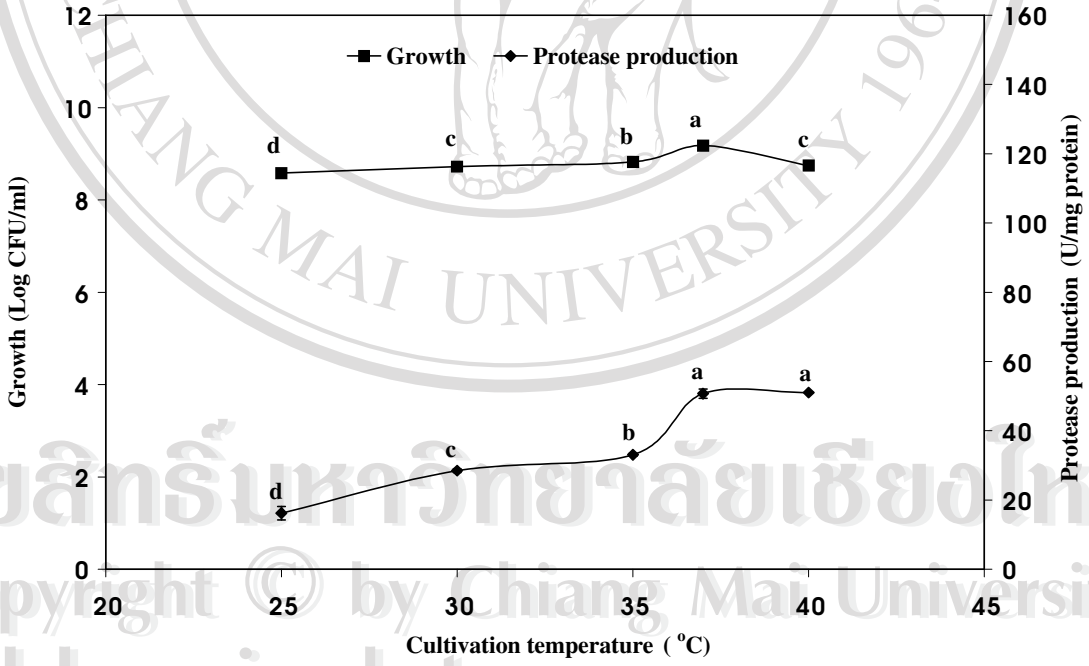

Figure 4.8 Effect of cultivation temperature on growth and protease production of *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations. Different letters (a,b,c,d) within the same parameter indicate significant differences (P<0.05).

4.2.4 Effect of agitation rate and ratio of medium to air content

To evaluate the effect of medium to air ratio and agitation rate on growth and protease production, the experiment was designed using 3×3 factorial with 3 agitation rates and 3 proportions of medium to air. Twenty, 40, and 60 ml of optimized medium were loaded to 125-ml flasks with the total volume of 160 ml to have the medium to air ratio at 1:7, 1:3 and 3:5, respectively. The flasks were incubated at 37°C for 24 h on a rotary shaker (100, 150 and 200 rpm). As illustrated in Figure 4.9., growth and protease production increased with increasing agitation rate and proportion of media to air. Highest growth and protease production was observed at proportion of media to air at 1:7 and rate of agitation rate at 200 rpm. The effect of oxygen supply on the protease production by altering the volume of the medium was investigated by Yang *et al*. (2000). The best production when the culture volume was 100 ml in a 250 ml Erlenmeyer flask with agitation rate 180 rpm. Chu *et al*. (1992) showed optimum yields of alkaline protease are produced at 200 rpm for *B. subtilis* ATCC 14416. The variation in the agitation speed and medium to air ratio influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. The production of protease by *Bacillus* sp. is an aerobic process, so dissolved oxygen tension (DOT) is an important factor. The reduction in the rate of aeration which results in lowering of DOT also gives lower enzyme yields (Milner *et al*., 1996).

4.2.5 Effect of inorganic salts

A 125-ml Erlenmeyer flask containing 20 ml of optimized medium from 4.2.2 was supplemented with 1.5% (w/v) $CaCl_2$ with/without 0.5% (w/v) $CuSO_4$, $FeSO_4$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$. The culture media was inoculated with 0.2 ml of the 15-h seed and incubated at 37°C for 24 h on a rotary shaker (200 rpm). The influence of inorganic salts on growth and protease production of *Bacillus* sp. FAS001 is shown in Table 4.1. Addition of inorganic salt did not decreased the growth. However, addition of $CaCl_2$ resulted in an increase in protease production, approximately 43.57% compared to basal medium. In addition to $CaCl_2$, the presence of other divalent ions inhibited protease production. $CuSO_4$ inhibited both growth and protease production, whereas $ZnSO_4$ and $FeSO_4$ severely inhibited protease production. The results were in agreement with Yang

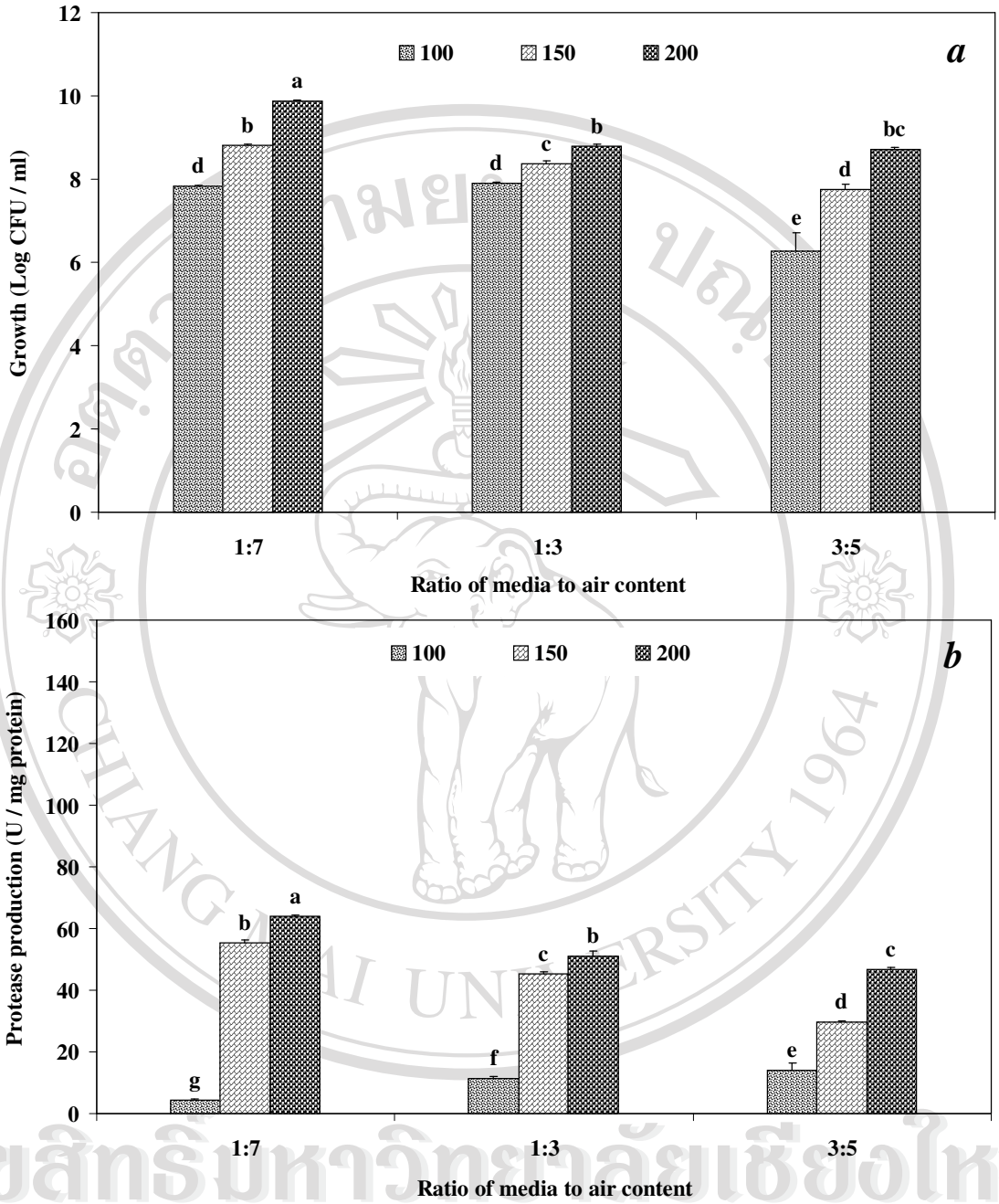

Figure 4.9 Effect of shaking rate and ratio of media to air content on growth (*a*) and protease production (*b*) of *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations. Different superscripts (a,b,c,d,e,f,g) within the same parameter indicate significant differences ($P<0.05$).

et al. (2000) that showed the optimal protease production was observe when 1.5% $CaCl_2$ was supplemented along with KH_2PO_4 and $MgSO_4$. Metal ion enhanced the production and stabilized the protease production (Janssen *et al*., 1994). Benjaree *et al*. (1999) showed that enzyme production was improved when calcium acetate, $CaCl_2$ and $MgSO_4$ were supplemented in the medium along with KH_2PO_4. The increase in enzyme production was small when these salts were used individually. Enzyme production in the absence of these salts was sufficiently low, thereby indicating the requirement of acetate and some metal ions (Ca^{2+} and Mg^{2+}). Neutral protease are optimally active near a pH of about 7.0, and their activity shows as absolute dependence on the presence of divalent metal ions (Matsubara and Feder, 1971). Brücker et al. (1990) suggested that the serine protease from *Bacillus subtilis* requires Ca^{2+} for stability.

Table 4.1 Effect of inorganic salt on growth and protease production of *Bacillus* sp. FAS001

Inorganic salt	Growth (Log CFU/ml)	Protease production (U/mg protein)
Basal (B)	8.88 ± 0.01^{a}	75.41 ± 5.69^{c}
B+1.5%$CaCl_2$	8.83 ± 0.01^{ab}	108.27 ± 4.61^{a}
B+1.5%$CaCl_2$+0.5%$CuSO_4$	ND	ND
B+1.5%$CaCl_2$+0.5%$FeSO_4$	8.82 ± 0.02^{b}	9.75 ± 4.91^{d}
B+1.5%$CaCl_2$+0.5%$MgCl_2$	8.80 ± 0.02^{b}	72.57 ± 1.84^{c}
B+1.5%$CaCl_2$+0.5%$MnCl_2$	8.84 ± 0.04^{ab}	84.88 ± 5.84^{b}
B+1.5%$CaCl_2$+0.5%$ZnCl_2$	8.82 ± 0.02^{b}	7.46 ± 0.46^{d}

Mean values with different superscripts (a,b,c,d) in the same column indicate significant differences ($P<0.05$); ND = Not detectable

4.2.6 Effect of media additives

A 125-ml Erlenmeyer flask containing 20 ml of optimized medium from 4.2.2 was added with 1.0% (w/v) bacto-peptone, corn flour, corn steep liquor, meat extract, molasses, rice bran, rice flour and yeast extract. The culture media was inoculated with 0.2 ml of the 15-h seed and incubated at 37°C for 24 h on a rotary shaker (200 rpm).Table 4.2 showed addition media additives had no significant effect on growth ($P <0.05$). Addition of complex media additives such as corn flour, rice flour and rice

bran resulted in an increase in protease production approximately 8.88%, 27.28% and 2.64%, respectively. In contrast, addition of common culture media including bacto-peptone, corn steep liquor, meat extract and yeast extract decreased the protease production. The protease production was highly inhibited in the presence molasses. The finding was contrasted to Fujiwara and Yamamoto (1987) that the addition of polypeptone and yeast extract to soybean meal significant increased the protease production. The results indicated that rice flour at 1% (w/v) was effective in stimulating protease production. It might be concluded that the hydrolytic activities of the *Bacilus* sp. strain are regulated by different induction levels depending on the composition of the medium. When correlated to previous works (Zouari *et al*., 1998; Sikdar *et al*., 1991; Goldberg *et al*., 1980), it was clear that the increase of the substrate complexity causes increase of hydrolytic activities. In crude gruel, a starch- and gluten-rich product, proteases were secreted in the logarithmic growth phase to supply nitrogen and amino-acids for the microorganism, and at the end of sporulation, intracellular proteases were released in the medium. This could explain the rise observed in protease activity at the end of the fermentation.

Table 4.2 Effect of media additive on growth and protease production of *Bacillus* sp. FAS001.

Media Additive	**Growth (Log CFU/ml)**	**Protease production (U/mg Protein)**
Basal (B)	8.88 ± 0.01^{a}	75.41 ± 5.69^{c}
B+bacto peptone	8.83 ± 0.02^{a}	50.24 ± 2.11^{d}
B+corn flour	8.77 ± 0.03^{a}	82.75 ± 5.71^{b}
B+corn steep liquor	8.82 ± 0.02^{a}	52.21 ± 0.41^{d}
B+meat extract	8.80 ± 0.02^{a}	51.66 ± 0.42^{d}
B+molass	8.84 ± 0.04^{a}	29.26 ± 1.37^{e}
B+rice bran	8.80 ± 0.02^{a}	77.45 ± 2.36^{c}
B+rice flour	8.81 ± 0.02^{a}	94.65 ± 1.22^{a}
B+yeast extract	8.77 ± 0.04^{a}	52.68 ± 2.28^{d}

Mean values with different superscripts (a,b,c,d,) in the same column indicate significant differences ($P<0.05$).

4.2.7 Effect of inoculum levels

A 125-ml Erlenmeyer flask containing 20 ml of optimized medium from 4.4.2. inoculated with different levels ranging from 1 to 5.0% (v/v) of the 15-h seed and incubated at 37°C for 24 h on a rotary shaker (200 rpm). The effect of inoculum levels on growth and protease production is shown in Figure 4.10. Growth and protease production increased with increasing of inoculum levels Seeding at 5.0% (v/v) had higher growth and protease production than 1.0 and 2.0%, but there were no significant differences with seeding at 3.0%. Therefore, seeding at 3.0% was selected for the next step. For maximum protease production, seeding of *Bacillus* sp. varied from 1 to 4% (Joo *et al*., 2002; Kanekar *et al*., 2002; Mehrotra *et al*., 1999; Raja, *et al*. 1994, Nehete *et al*.,1986). Importance of inoculum size on microbial fermentation processes is widely accepted. It is clear that the protease production steadily increased with the increasing size of inoculum until it reached the magnitude when enzyme productivity became maximum. Thereafter no appreciable change in enzyme production with higher inoculum size could be observed. This indicates that the inoculum density do have limited effect on fermentation processes. It has some optimum value depending on the microbial species and fermentation system

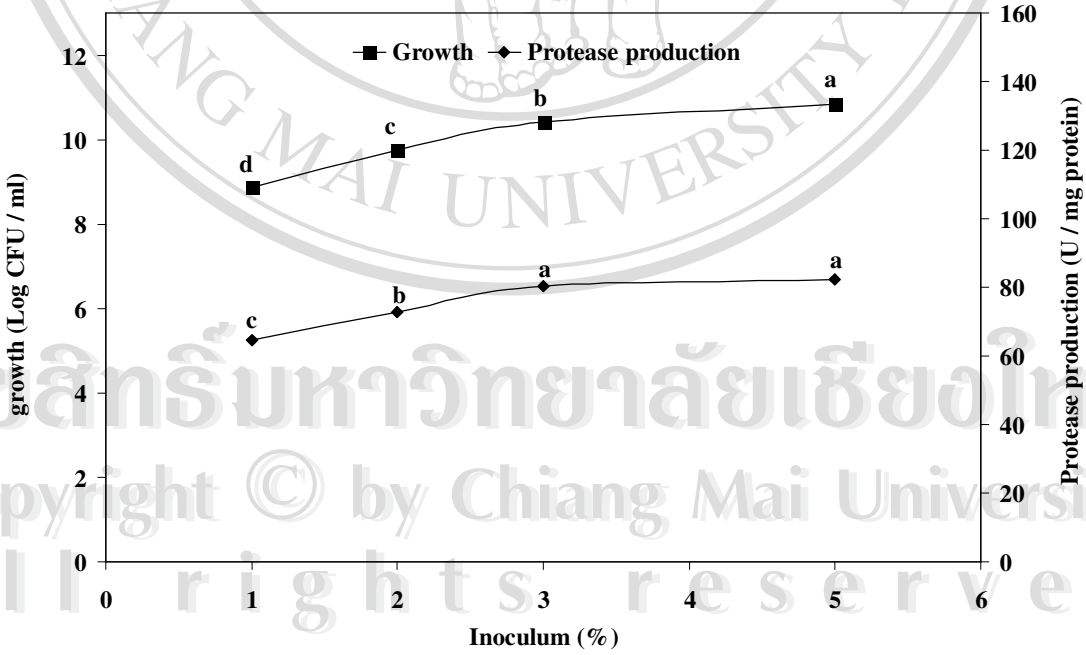

Figure 4.10 Effect of inoculum on growth and protease production of *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations. Different superscripts (a,b,c,d) within the same parameter indicate significant differences (P<0.05).

4.2.8 Time course of enzyme production

A 125-ml Erlenmeyer flask containing 20 ml of optimized medium from 3.2.4.5 and 3.2.4.6 was inoculated with 0.6 ml of the 15-h seed and incubated at 37°C for 24 h on a rotary shaker (200 rpm). Time-course of growth and protease production are shown in Figure 4.11. Similar growth profiles were observed in basal medium (B) and basal medium + $CaCl_2$ 1.5% (w/v) in which a maximum growth was observed at 24 h. However, the addition of 1.0% (w/v) rice flour and 1.5% (w/v) $CaCl_2$ accelerated growth and resulted in an increase protease production. It was estimated that protease production under optimal condition (Table 4.3) was about 3 times higher than that of initial condition. The production of an enzyme exhibits a characteristic relationship with regard to the growth phase of that organism. In general, the synthesis of protease in *Bacillus* species is constitutive or partially inducible and is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phase (Priest, 1977; Strauch and Hoch 1993). The production of extracellular proteases during the stationary phase of growth is characteristic of many bacterial species (Priest, 1977). At early stationary phase, two or more proteases are secreted and the ratio of the amount of the individual proteases produced also varied with the *Bacillus* strains (Priest, 1977; Uehara *et al*., 1974). In several cases, the function of the enzyme is not very clear, but its synthesis is correlated with the onset of a high rate of protein turnover and often sporulation (Chu *et al*., 1992).

Table 4.3 The optimum medium contents and cultural conditions for protease production of *Bacillus* sp. FAS001.

Optimized cultural medium		**Optimized cultural condition**	
SBM	2%	pH	5.0
K_2HPO_4	0.1%	Temperature	37°C
$MgSO_4$	0.05%	Media to air content	1:7
Rice flour	1.0%	Agitation rate	200 rpm
$CaCl_2$	1.5%	Inoculum levels	3%
		Time	36 h
Protease production	**140 U / mg protein**		

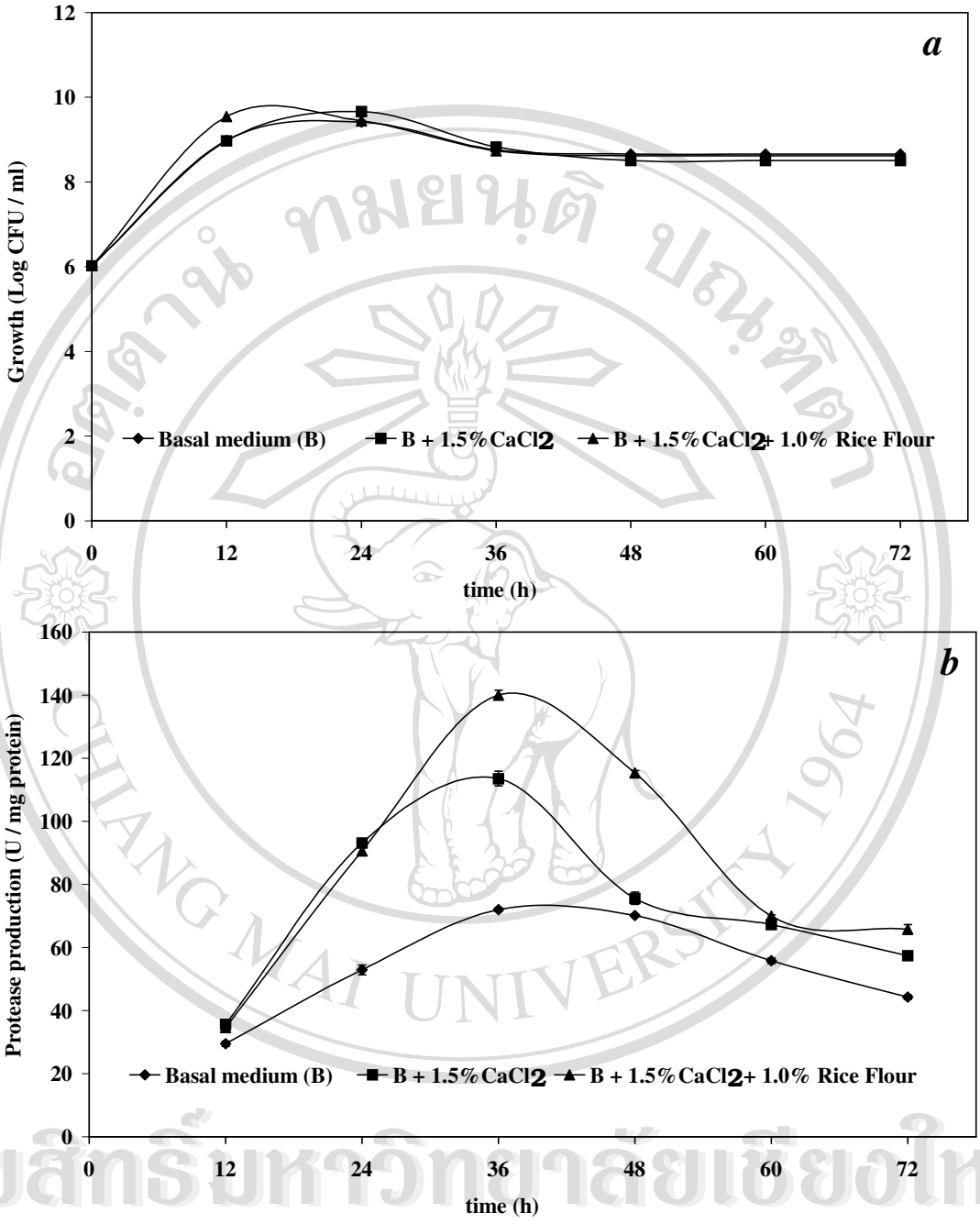

Figure 4.11 Time cause on growth (a) and protease production (b) of *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations.

4.2.9 Production of crude protease powder

Crude spent cell culture broth obtained from liquid fermentation was mixed with various types of carriers and dried at 50°C for 6 h. Table 4.4 shows activity of crude protease on different carriers. Based on the same amount of enzyme added at the beginning, corn flour retained the highest protease activity after drying followed by rice flour ($P<0.05$). Compared to corn flour, SBM, rice bran, and SPI were found to be ineffective in preventing protease from heat inactivation.

Table 4.4 Effect of carriers on the activity of crude protease powder

Carriers	Activity (U/g)
Corn Flour	727.09[a]
Rice bran	247.70[c]
Rice flour	579.35[b]
Soybean meal	233.45[c]
Soy protein isolate	126.92[d]

Mean values with different superscripts (a,b,c,d,) in the same column indicate significant differences ($P<0.05$). Activity was assayed at pH 6.8, 39.5°C for 10 min by using soy protein isolate as substrate.

4.3 Effect of temperature, time, and air exposure during storage

As shown in Figure 4.12, the activity of unopened crude protease powder stored at both 4°C and room temperature remained stable during experimental period (12 months). Storage at 45°C resulted in a significant decrease in activity after 2 weeks in which the activity retained about 80% of initial activity. Similar changes in protease activity were obtained in left over product which was opened at the beginning. The results suggested that crude protease powder was stable with no effect of air exposure after opening. Changes in activity of imported enzyme (IE1) are shown in Figure 4.13. Protease activity of both unopened and opened IE1 decreased with the increasing storage temperature and time. With the exception, unopened IE1 stored at

4°C showed little decrease in the activity. The results suggested that crude protease powder exhibited better storage ability than IE1. Normally enzyme must be kept at low temperature to avoid undesired effect. How storage condition affect enzyme activity

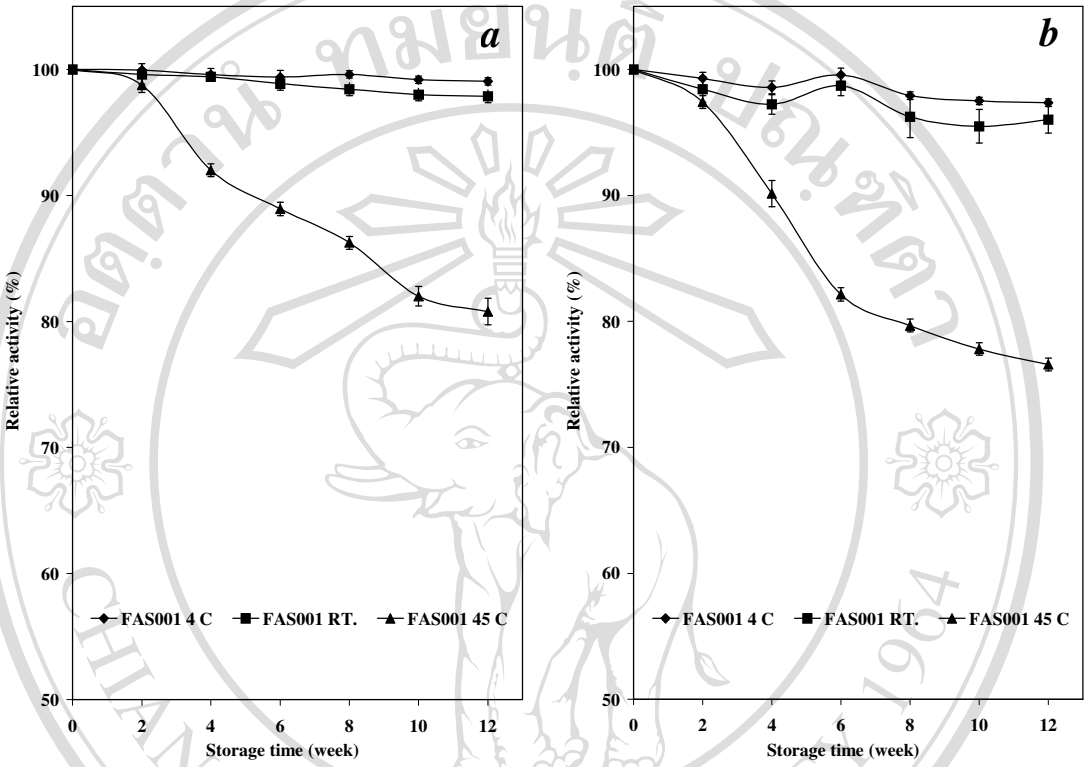

Figure 4.12 Effect of temperature and time on protease activity of unopened (*a*) and opened (*b*) protease powder from *Bacillus* sp. FAS001 during storage; Bars indicate standard deviations from 3 determinations.

4.4 Characterization of crude protease powder from *Bacillus* sp. FAS001

4.4.1 Optimum pH

The pH-activity profile of the enzyme is showed in Figure 4.14. Maximum enzyme activity was observed at pH 6.0 and an increase in pH beyond 6.0 brought about a decline in protease activity resulting in 50% activity at pH 8.0. At pH 3.0, 65% of the maximum enzyme activity was obtained, increasing to 72 and 74% at pH 4.0 and 5.0, respectively. These characteristics showed that crude protease powder of *Bacillus* sp. FAS001 was active in a broad pH ranges. For feed supplement, enzyme can act either in stomach (pH 3.0) or small intestine (pH 6.8).

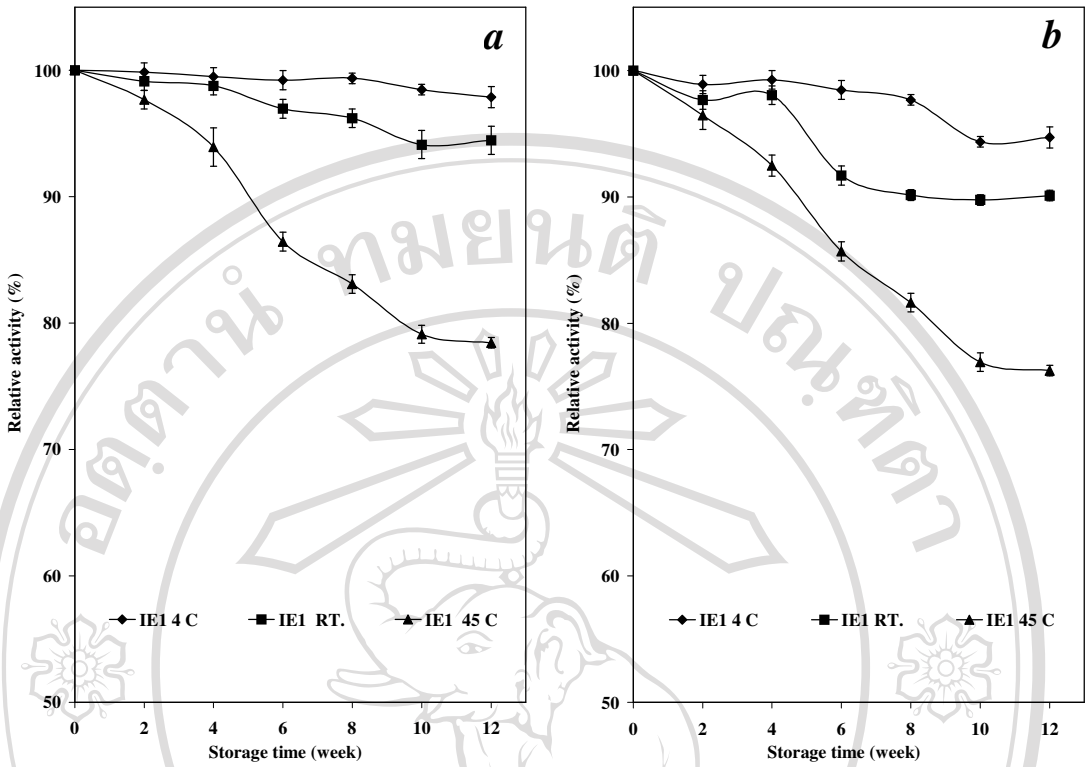

Figure 4.13 Effect of temperature and time on protease activity of unopened (*a*) and opened (*b*) imported enzyme (IE1) during storage; Bars indicate standard deviations from 3 determinations.

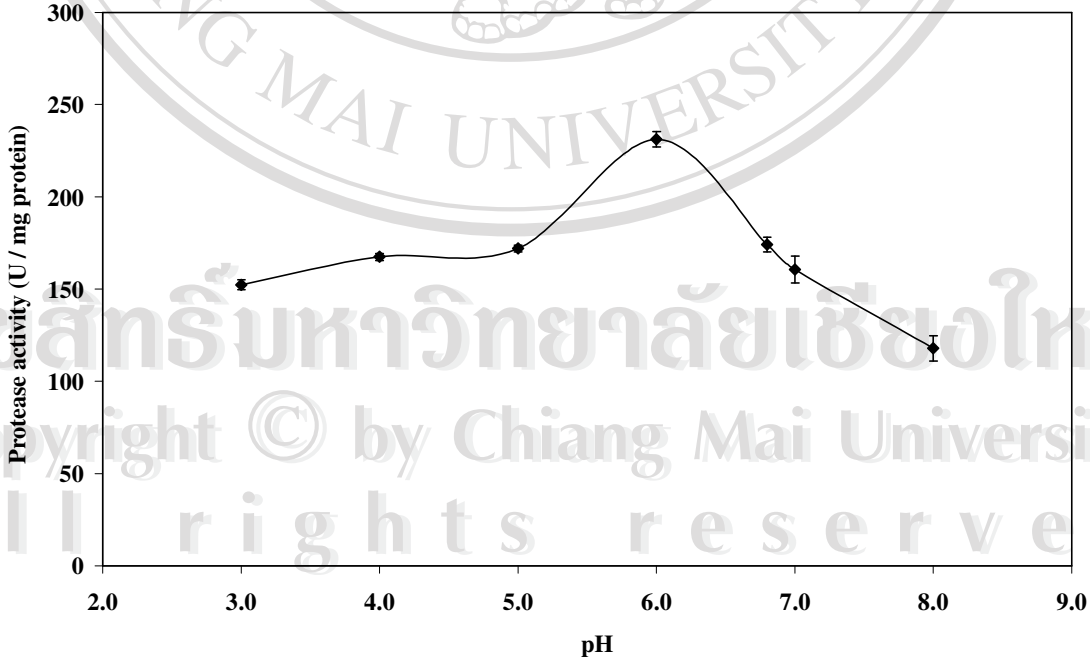

Figure 4.14 Effect of pH on the activity of crude protease from *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations.

4.4.2 Optimum temperature

Maximum protease activity was observed at 55°C (Figure 4.15). Enzyme activity decreased at temperatures higher than 60°C. It is reported that a number of alkaline proteases isolated from *Bacillus* sp. have high optimal temperatures for their activity.

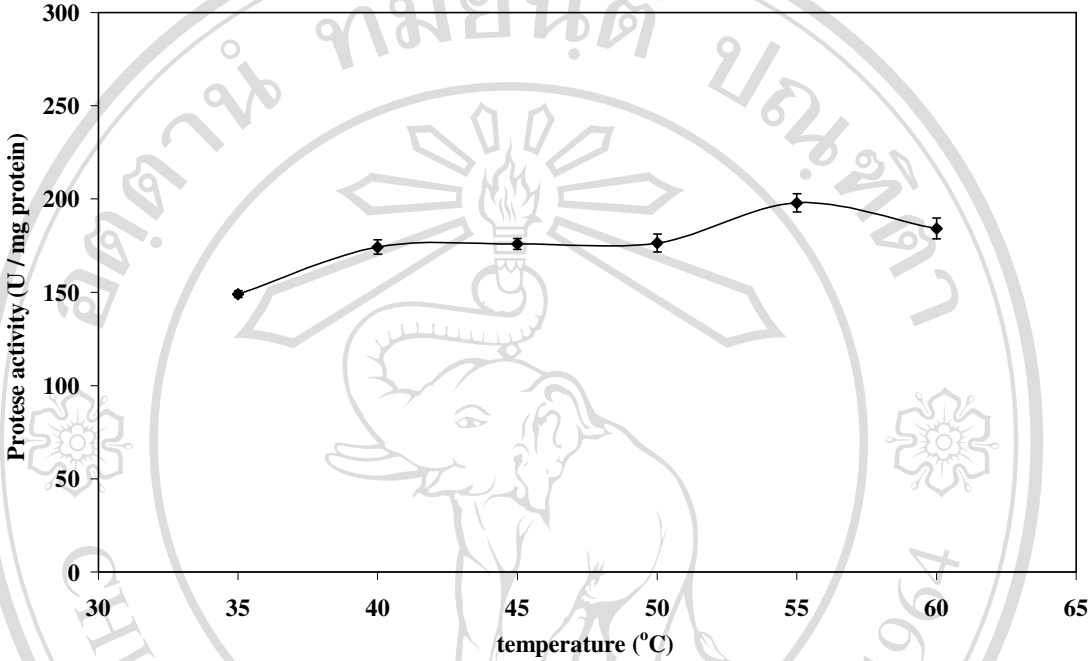

Figure 4.15 Effect of temperature on the activity of crude protease from *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations.

4.4.3 Activity profiles

Compared to commercial feed enzymes, IE1 and IE2, crude protease powder of *Bacillus* sp. FAS001 showed highest protease activity at both pH 3.0 and 6.8 (Figure 4.16). In addition to protease, crude enzyme preparation of *Bacillus* sp. FAS001 also contained other carbohydrate hydrolyzing enzymes such as amylase, β-glucanase, cellulase, pentosanase and phytase. Except protease, IE1 and IE2 contained much higher activity of these enzymes than crude protease powder. The present of other enzyme activities especially carbohydrase, protease and phytase. Carbohydrase (β-glucanase, cellulase, pentosanase and amylase) are primary against the non-starch polysaccharide (NSP) and starch compositions (Partridge, 2000). Phytase is very effective for improving the availability of phytate phosphorus resulting in enhanced the bioavailability of minerals, amino acid/protein and even starch (Kornegay, 2000). The previous studies showed that *Bacillus* sp. are capable of producing the enzyme

not only protease but also other carbohydrases and phytase. (Kerovuo *et al*., 1998; Lin *et al*., 1998; Yang *et al*., 1995; Ozaki *et al*., 1990).

4.4.4 Cytotoxicity test

The MTT assay was the rapid method for determining the potential toxicity of compounds. As illustrated in Table 4.5, within ± 10% of control, culture media alone and crude protease powder was considered no toxicity to the tested cell lines over the range of 100-200 µg/ml. However, lower percentage of survival was observed in samples containing bacterial cells. *Bacillus* is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low. However, similar to other closely related species in the genus such as *B. licheniformis, B. pumulis*, and *B. megaterium* and *B. subtilis* have been shown to be capable of producing lecithinase, an enzyme which disrupts membranes of mammalian cells. In addition, it also possibly produce an extracellular toxin known as subtilisin that use in laundry detergent products. There have been a number of cases of allergic or hypersensitivity reactions, including dermatitis and respiratory distress after the use of these laundry products (Norris *et al*.,1981).

Table 4.5 Cell cytotoxicity of cell culture and crude protease powder from *Bacillus* sp. FAS001.

	Cell cytotoxicity [1]			
	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
Cell culture				
Nutrient broth (NB)	ND	ND	98	93
Bacillus sp. FAS001 with NB	ND	ND	77	78
SBM medium	ND	ND	102	104
Bacillus sp. FAS001 with SBM medium	ND	ND	88	83
Crude protease				
Bacillus sp. FAS001	109	108	95	99

[1] Cell cytotoxicity was represent as the percentage of survival compared to control; ND = Not determined

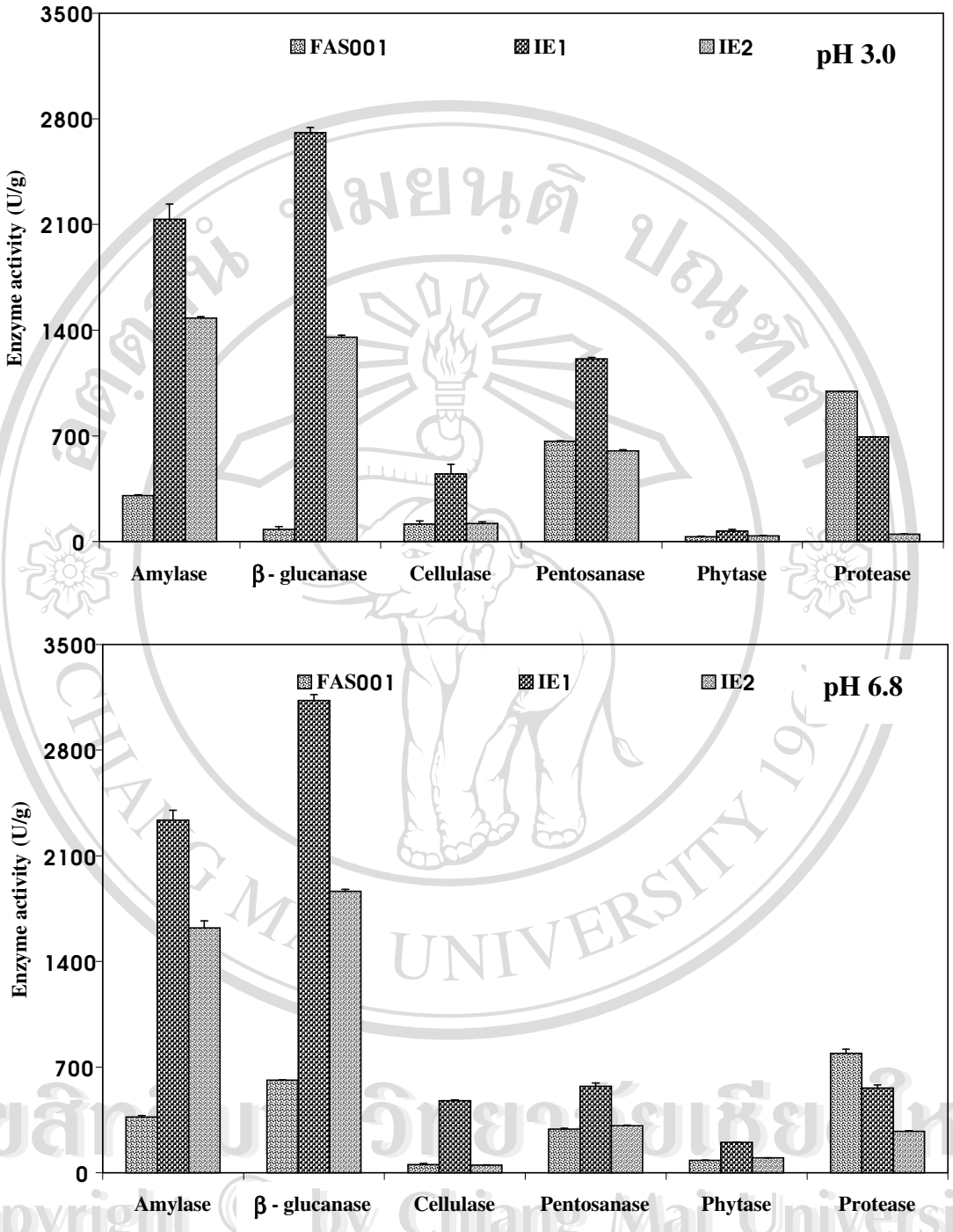

Figure 4.16 Activity profiles of crude protease from *Bacillus* sp. FAS001; Bars indicate standard deviations from 3 determinations.

Table 4.5 Cell cytotoxicity of cell culture and crude protease powder from *Bacillus* sp. FAS001.

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Crude protease				
Bacillus sp. FAS001	109	108	95	99

[1] Cell cytotoxicity was represent as the percentage of survival compared to control; ND = Not determined

4.5 Testing of enzyme quality by *in vitro* digestibility

The *in vitro* nutrient digestibility of all diets tested is shown in Table 4.6. Among all diets, piglet diet exhibited higher *in vitro* DM, CP, and NFE digestibility than other diet formulations ($P<0.05$). However, no differences in digestibility of CF, Ash, and EE were observed in all diets. Based on the formulation (Table 3.2), the results clearly indicated that *in vitro* digestibility of diet largely depended on the complex nature of the feed ingredients used. The use of full fat soybean meal, soybean meal and nucospray K10 contributed to higher DM, CP, and NFE digestibility of piglet diet. The results suggested that replacing or lowering the proportion of these ingredient with ground corn, cassava meal and rice bran substantially decreased the *in vitro* CP digestibility (from 77 to 60%). These differences in CP digestibility between tested diets can possibly be explained by differences in the gross chemical composition of the grains especially NSP. NSP act as a physical barriers to digestive enzyme, such as amylase and protease, thus reducing their efficient digestion (Lin *et al*., 1998). An increase in viscosity digesta decreased the rate of diffusion of substrate-digestive enzyme so that

enzyme can not penetrate into the system. Furthermore, an increased in protein digestibility may be due to the presence of protease inhibitor in ground corn, cassava meal and rice bran. (Birk, 1989).

Compared to the control (with no enzyme added), the enzyme supplement (IE1, IE2, and FAS001) resulted in higher *in vitro* digestibility of all nutrients in all diets tested ($P<0.05$). At the same level of enzyme added, crude protease powder FAS001 and IE1 were more effective than IE2 in improving *in vitro* nutrient digestibility of all diets tested. Compared to piglet diet, enzyme supplement resulted in a larger increase in the *in vitro* nutrient digestibility of growing piglet, finishing piglet, and pregnant piglet diets. Based on the same protease activity added, protein digestibility of all diets increased about 3-5% of those observed in control in which the protein digestibility of FAS001 diet was comparable with IE1 diet but higher than IE2 diet. However, it should be noted that supplement with crude protease powder FAS001 resulted in much larger increases in digestibility of DM (~10-11%) and NFE (~15-16%). Besides protease, this was possibly caused by the presence of other enzymes, particularly pentosanase and carbohydraes detected in the crude protease powder (Figure 4.17). Solubilization of the polysaccharides by pentosanase resulted in releasing of starch and protein from the cell wall structure to make them better available for the digestive system. The exogenous amylase can effectively the endogenous to digest starch component. Furthermore, the presence of phytase can decreased the effect of phytate on the binding with minerals starch and proteins(Birk, 1989). With an increasing concentration of enzyme added, FAS001 1.5X diet of all formulations showed highest digestibility of all nutrients. Compared to those of control, DM, CP and NFE digestibility increased to approximately 15%, 9% and 20%, respectively.

Table 4.6 *In vitro* digestibility of dry matter (DM), crude protein (CP), crude fibre (CF), ether extract (EE), ash and nitrogen free extract (NFE) in four different diets.

	Treatments[1]				
	Con	**IE 1**	**IE 2**	**FAS 001 1.0X**	**FAS 001 1.5X**
Piglet diet					
DM	68.52^{e}	71.54^{c}	70.51^{d}	72.46^{b}	75.61^{a}
CP	76.60^{e}	79.75^{c}	78.54^{d}	81.16^{b}	82.54^{a}
CF	50.28^{d}	52.54^{b}	51.43^{c}	52.84^{b}	53.48^{a}
Ash	51.54^{d}	53.43^{b}	52.37^{c}	53.27^{b}	54.31^{a}
EE	50.44^{d}	52.20^{b}	51.32^{c}	52.42^{b}	53.85^{a}
NFE	74.62	78.08	77.18	80.82	84.36
Growing pig diet					
DM	65.61^{d}	69.40^{bc}	68.55^{c}	72.46^{b}	75.47^{a}
CP	60.20^{d}	63.36^{b}	62.08^{c}	63.56^{b}	65.66^{a}
CF	50.55^{d}	52.48^{b}	51.66^{c}	52.74^{b}	53.40^{a}
Ash	51.45^{d}	53.74^{b}	52.46^{c}	53.44^{b}	54.31^{a}
EE	50.78^{d}	52.48^{b}	51.44^{c}	52.60^{b}	53.27^{a}
NFE	72.22	77.24	76.63	82.93	87.42
Finishing pig diet					
DM	65.44^{d}	69.43^{bc}	68.61^{c}	72.47^{b}	75.40^{a}
CP	60.45^{d}	63.51^{b}	62.81^{c}	63.69^{b}	66.35^{a}
CF	50.50^{d}	52.29^{b}	51.53^{c}	52.88^{b}	53.50^{a}
Ash	51.68^{d}	53.65^{b}	52.44^{c}	53.35^{b}	54.56^{a}
EE	50.44^{d}	52.45^{b}	51.39^{c}	52.70^{b}	53.63^{a}
NFE	72.91	78.21	77.43	83.58	87.58
Pregnant pig diet					
DM	65.36^{e}	69.48^{c}	68.43^{d}	72.54^{b}	75.50^{a}
CP	60.29^{c}	63.10^{ab}	62.55^{b}	63.57^{a}	65.64^{a}
CF	50.59^{c}	52.53^{a}	51.44^{b}	52.54^{a}	53.83^{a}
Ash	51.35^{c}	53.28^{a}	52.38^{b}	53.53^{a}	54.62^{a}
EE	50.19^{d}	52.21^{b}	51.61^{c}	52.74^{b}	53.50^{a}
NFE	71.84	77.87	76.47	83.51	88.04

[1] Con = Control, IE1 = Imported Enzyme 1, IE 2 = Imported Enzyme 2 (same protease activity as IE1), FAS001 1.0X = Crude protease (same protease activity as IE1) and FAS001 1.5X = Crude protease (1.5 times of protease activity with IE1).

Mean values with different superscripts (a,b,c,d,) in the same row indicate significant differences ($P<0.05$).

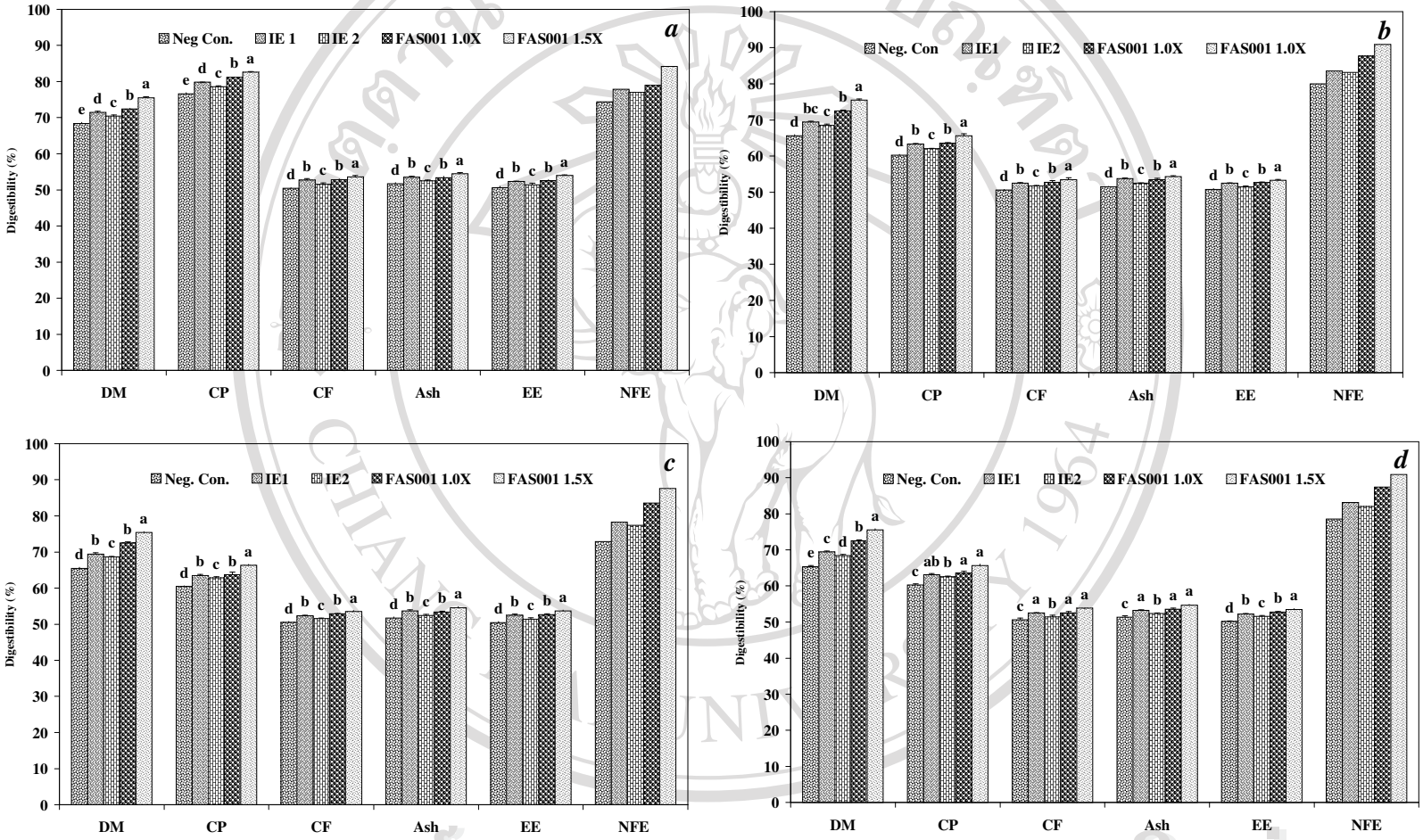

Figure 4.17 *In vitro* digestibility of nutrients (DM, CP, CF, EE, Ash and NFE) of piglet (*a*), growing pig (*b*), finishing pig (*c*) and pregnant pig (*d*) diets with or without enzymes; Bars indicate standard deviations from 3 determinations. Mean values with different superscripts (a,b,c,d,e) in the same column indicate significant differences (P<0.05).

4.6 Testing effect of enzyme on production performance in pigs

Data on average initial weight, average total feed intake, average final weight, average daily feed intake (ADFI), average daily gain (ADG), feed conversion ratio (FCR) and feed cost per gain (FCG) are presented in Table 4.7. It was summarized that enzyme supplementation enhanced pig performance in terms of average weight gain and FCR. Based on the similar initial weight, a final weight of enzyme supplemented group was higher than control group. As a result, FAS001 1.5X showed the lowest feed cost per gain, followed by FAS001 1.0X, IE2, IE1 and control, respectively. As illustrated in Table 4.8, ADFI of piglets were not significantly different across treatments ($P>0.05$). With the same level of enzyme addition, FAS001 1.0X had higher ADG than IE1 and IE2 for all periods. In Week 1-4, IE1 showed the same ADG as IE2. However, in week 5-6, IE1 had higher ADG than IE2. In the overall (week 1-6), IE1 had higher ADG than IE2 but not significant difference. The FCR value had same trend as ADG. The increase in FAS001 level from 1.0X to 1.5X showed highest ADG and FCR. Corresponded with the obtained results from the *in vitro* digestibility, enzyme supplement resulted in better production performance. In growth trials, it certainly appears that some of the benefits seen *in vitro* can be translated into improved productive performance, which could be of particular value to the pig. Rooke *et al*. (1996) showed showed clear benefits in growth performance by the use of soybean meal treated with protease in diets for weaned pigs. The protease supplement resulted in hydrolysis the protein in soybean into the lower molecular weight units (Beal *et al*., 1998c), coupled with its effects on the proteinaceous anti-nutrients (trypsin inhibitor, lectins). Beside direct effect of protease , the presence of pentosanase in crude protease powder alleviate the effect of NSP on the protein digestion by decreasing loss of epithelial cells (Russett, 1998).

Table 4.7 Production performance and feed cost of piglets offered diets with or without enzymes.

Parameters		Treatments[1]				
		Con	IE 1	IE 2	FAS001 1.0x	FAS001 1.5x
Average initial weight	(kg)	4.94[a]	4.97[a]	4.98[a]	4.98[a]	5.04[a]
Average final weight	(kg)	19.64[e]	20.25[c]	20.06[d]	20.70[b]	21.44[a]
Average weight gain	(kg)	14.70[e]	15.28[c]	15.08[d]	15.72[b]	16.40[a]
Average total feed intake	(kg)	23.07[a]	23.09[a]	22.91[a]	22.57[a]	22.92[a]
Total feed cost	(Baht)	442.12	445.74	442.81	439.49	443.23
Feed cost per gain	(Baht /kg)	30.08	29.18	29.36	27.96	27.02

[1] Con = Control, IE1 = Imported Enzyme 1, IE 2 = Imported Enzyme 2 (same protease activity as IE1), FAS001 1.0X = Crude protease (same protease activity as IE1) and FAS001 1.5X = Crude protease (1.5 times of protease activity with IE1).

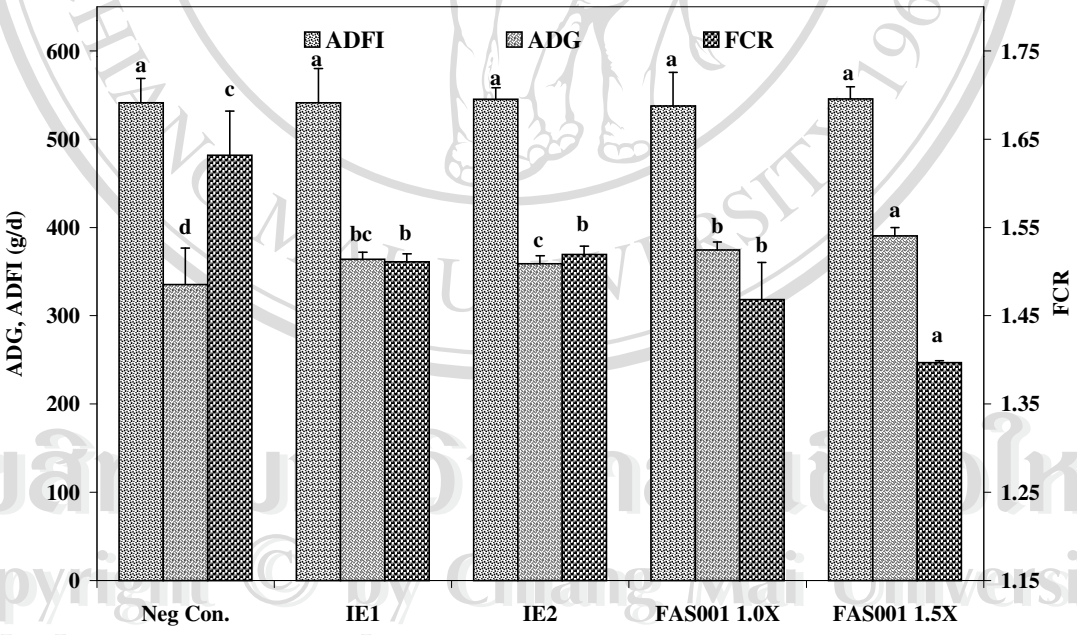

Figure 4.18 Growth performance of piglets given diets with or without enzymes; Bars indicate standard deviations from 16 replications. Mean values with different letters (a,b,c,d,) in the same parameter indicate significant differences (P<0.05).

Table 4.8 Growth performance of piglets offered diets with or without enzyme.

		Treatments [1]				
		Con	**IE1**	**IE2**	**FAS001 1.0X**	**FAS001 1.5X**
ADFI.	(g / d)					
Week 1		190.63^{ab}	193.23^{a}	188.62^{b}	188.17^{b}	189.21^{b}
Week 2		372.99^{a}	374.63^{a}	372.54^{ab}	369.79^{b}	371.35^{b}
Week 3		533.48^{a}	530.13^{ab}	526.56^{b}	528.57^{b}	527.98^{b}
Week 4		631.70^{ab}	634.82^{a}	629.91^{bc}	625.97^{c}	629.99^{bc}
Week 5		741.29^{a}	739.06^{a}	735.04^{b}	734.97^{b}	734.08^{b}
Week 6		826.04^{a}	826.93^{a}	820.09^{b}	824.26^{ab}	821.80^{b}
Week 1-6		541.26^{a}	541.37^{a}	545.46^{a}	537.46^{a}	545.73^{a}
ADG .	(g / d)					
Week 1		103.57^{a}	95.83^{b}	93.30^{b}	93.01^{b}	95.54^{b}
Week 2		232.37^{c}	238.10^{c}	232.37^{c}	245.01^{b}	254.76^{a}
Week 3		342.19^{d}	355.36^{c}	361.38^{c}	373.59^{b}	399.93^{a}
Week 4		483.93^{d}	508.26^{c}	511.61^{c}	521.80^{b}	548.81^{a}
Week 5		491.82^{d}	504.91^{c}	494.20^{d}	513.32^{b}	531.18^{a}
Week 6		446.73^{e}	480.51^{c}	461.25^{d}	499.40^{b}	513.69^{a}
Week 1-6		335.57^{d}	363.83^{bc}	359.02^{c}	374.36^{b}	390.65^{a}
FCR						
Week 1		1.85^{a}	2.02^{b}	2.02^{b}	2.06^{b}	1.98^{b}
Week 2		1.61^{c}	1.58^{c}	1.61^{c}	1.51^{b}	1.46^{a}
Week 3		1.56^{d}	1.49^{c}	1.46^{c}	1.41^{b}	1.32^{a}
Week 4		1.31^{d}	1.25^{c}	1.23^{c}	1.20^{b}	1.15^{a}
Week 5		1.51^{d}	1.46^{c}	1.49^{d}	1.43^{b}	1.38^{a}
Week 6		1.85^{e}	1.72^{c}	1.78^{d}	1.65^{b}	1.60^{a}
Week 1-6		1.63^{c}	1.51^{b}	1.52^{b}	1.47^{b}	1.40^{a}

[1] Con = Control, IE1 = Imported Enzyme 1, IE 2 = Imported Enzyme 2 (same protease activity as IE1), FAS001 1.0X = Crude protease (same protease activity as IE1) and FAS001 1.5X = Crude protease (1.5 times of protease activity with IE1).

Mean values with different superscripts (a,b,c,d,e) in the same row indicate significant differences ($P<0.05$).

The illness rate and mortality rate are presented in Table 4.9. The illness rate by diarrhea of the pigs was found in week 1-3, the pigs fed IE2 diet shown the lowest illness rate in the first week. However, at week 2 and 3, the pig fed FAS001 1.0X and 1.5X tend to have the lowest illness rate among treatments. The impair digest in piglet showed loss in nutrient to large intestine that caused high proliferation of microbial especially *E. coli*. *E. coli* is the most important and most common cause of piglet diarrhea identified by diagnostic laboratories and veterinary practitioners (Steve 2000). Reducing the amount of protein by improved the immature digestive tract of the piglet with exogenous enzyme reduces the level of undesirable end products from protein digestion and minimizes the risk of diarrhea (PIC USA, 2003).

4.7 Effect on blood urea nitrogen (BUN)

Blood urea nitrogen (BUN) of pigs fed various diets are presented in Table 4.10. The pigs fed FAS001 1.5X diet had the lowest BUN among treatments at week 2, 4 and 6 ($P<0.05$). The comparison between the pigs on the IE1, IE2 and FAS001 1.0X diets, which were supplemented with the same level of activity shown that the FAS001 1.0X group had lowest BUN at week 2 and 6 ($P<0.05$). With the exception of the pigs fed IE1 diet had lowest BUN at week 4 ($P<0.05$). The blood urea nitrogen (BUN) is the end product of protein metabolism and its concentration is influenced by the rate of excretion. BUN indicates the amount of urea circulating in the system. BUN generally reflects how much nitrogen is being absorbed and used for growth compared to how much is being wasted and is burdening the system so it can be excreted. An animal that is being overfed nitrogen will have a higher BUN level and be a productive challenge. Kidney damage, certain drugs, low fluid intake, intestinal bleeding, exercise, or heart failure especially excessive protein intake can cause increases the BUN concentration. The high level of BUN indicated the excessive protein excrete by imbalance protein in diet. The enzyme supplement can improve the balance in amino acid pattern resulting in lowering BUN concentration.

Table 4.9 Illness and mortality rates of piglets offered diets with or without enzyme supplementation.

Week	Con		IE1		IE2		FAS001 1.0X		FAS001 1.5X	
	Illness rate	Mortality rate	Illness rate	Mortality rate	Illness rate	Mortality rate	Illness rate	Mortality rate	Illness rate	Mortality rate
1	20 (31.25)	0 (0.00)	20 (31.25)	0 (0.00)	17 (2656)	0 (0.00)	18 (28.13)	1 (1.56)	19 (29.69	1 (1.56)
2	100 (31.25)	1 (1.56)	(31.25)	1 (1.56)	(31.25)	1 (1.56)	(31.25)	0 (0.00)	(31.25)	0 (0.00)
3	12 (18.75)	0 (0.00)	10 (15.87)	0 (0.00)	10 (18.87)	0 (0.00)	5 (7.94)	0 (0.00)	6 (9.52)	0 (0.00)
4	9 (14.06)	1 (1.56)	4 (6.43)	0 (0.00)	5 (7.81)	0 (0.00)	3 (4.67)	0 (0.00)	3 (4.76)	0 (0.00)
5	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
6	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total		2 (3.13)		1 (3.13)		0 (0.00)		1 (1.56)		1 (1.56)

[1] Con = Control, IE1 = Imported Enzyme 1, IE 2 = Imported Enzyme 2 (same protease activity as IE1), FAS001 1.0X = Crude protease (same protease activity as IE1) and FAS001 1.5X = Crude protease (1.5 times of protease activity with IE1).

[2] Number in parenthesis is percentage of total pig (65).

Table 4.10 Blood urea nitrogen of piglets offered diets with or without enzyme

	Treatment [1]				
BUN (mg/dl)	**Con**	**IE1**	**IE2**	**FAS001 1.0X**	**FAS001 1.5X**
Initial	43.26^{b}	41.54^{a}	42.16^{b}	42.19^{b}	41.90^{b}
Week 2	41.61^{d}	31.00^{b}	32.41^{c}	32.63^{c}	29.88^{a}
Week 4	32.11^{d}	25.52^{c}	24.72^{c}	21.09^{b}	14.78^{a}
Week 6	39.85^{c}	29.77^{b}	30.07^{b}	30.40^{b}	24.62^{a}

[1] Con = Control, IE1 = Imported Enzyme 1, IE 2 = Imported Enzyme 2 (same protease activity as IE1), FAS001 1.0X = Crude protease (same protease activity as IE1) and FAS001 1.5X = Crude protease (1.5 times of protease activity with IE1).

Mean values with different superscripts (a,b,c,d,) in the same row indicate significant differences (P<0.05).

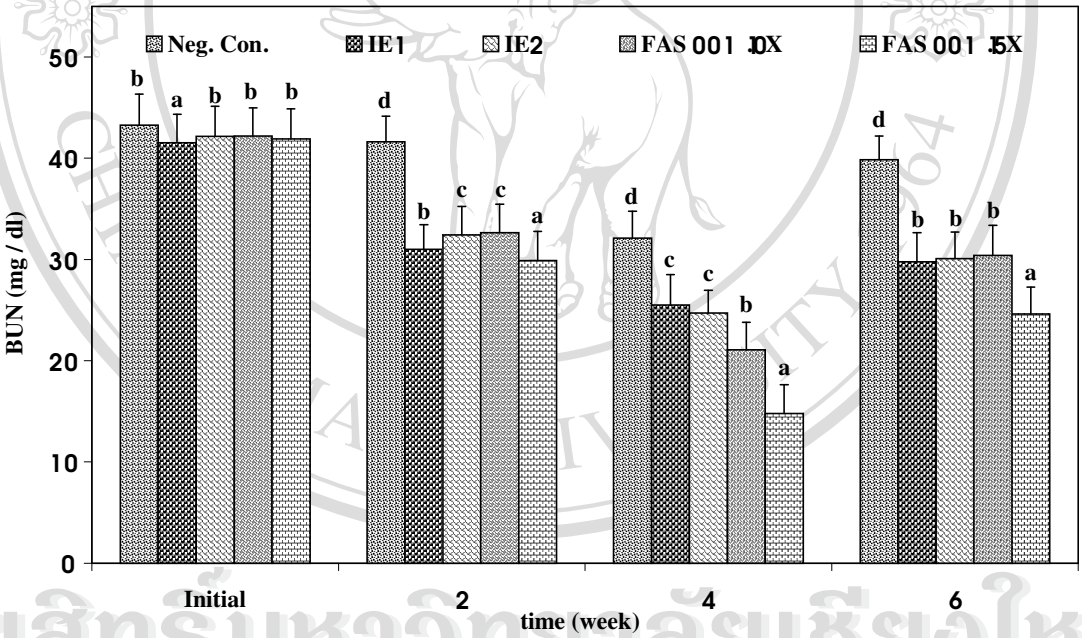

Figure 4.19 Blood urea nitrogen of piglets given diets with or without enzymes; Bars indicate standard deviations from 16 replications. Mean values with different letters (a,b,c,d,) in the same week indicate significant (P<0.05).