CHAPTER4

EXPERIMENTAL RESULTS AND DISCUSSION

Since it was previously discovered that ATPs of PEG 1000/ potassium phosphate gave higher alkaline protease partition coefficients (K_{AK}) than other systems of different PEG molecular weights (Sebastiao et al, 1996), PEG 1000 was first selected as polymer component in our extractive fermentations. As a result, PEG 1000/ phosphate ATPs were extensively investigated for extractive fermentation in the following section in order to determine suitable system compositions for alkaline protease production. However, it was later discovered that PEG 1000 was not appropriate for extractive fermentation, sections that follow, therefore, involve investigation for suitable PEG molecular weight. The chapter finishes with discussion on effect of PEG and potassium phosphate concentrations, and phase volume ratio on alkaline protease production.

4.1 Conventional fermentation:

Conventional fermentation was carried out for 71 hours, and the results are shown in figure 4.1, 4.2 and 4.3.

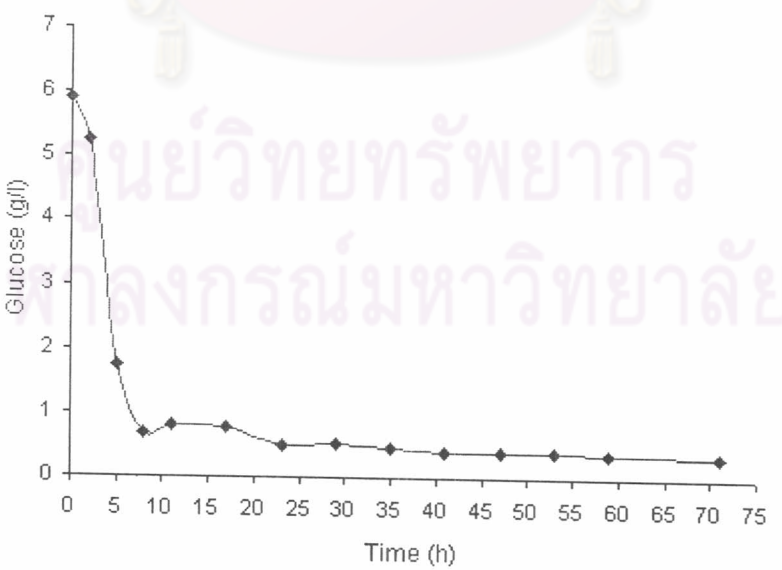

Figure 4.1: Time-course of glucose in conventional alkaline protease fermentation. Conditions: pH 7 and at 37 °C and at 250 rpm agitation,

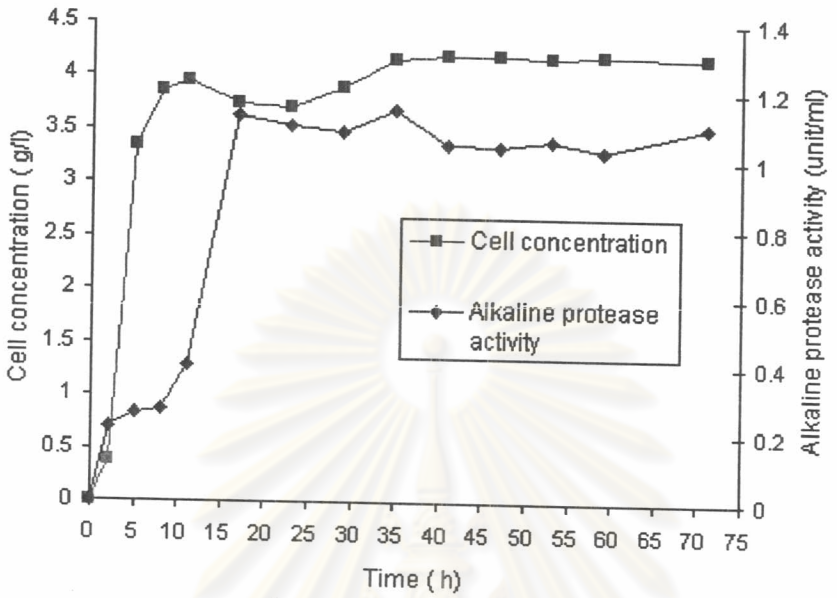

Figure 4.2: Time-course of cell and alkaline protease in conventional alkaline protease fermentation. Conditions: pH 7 and at 37 °C and at 250 rpm agitation,

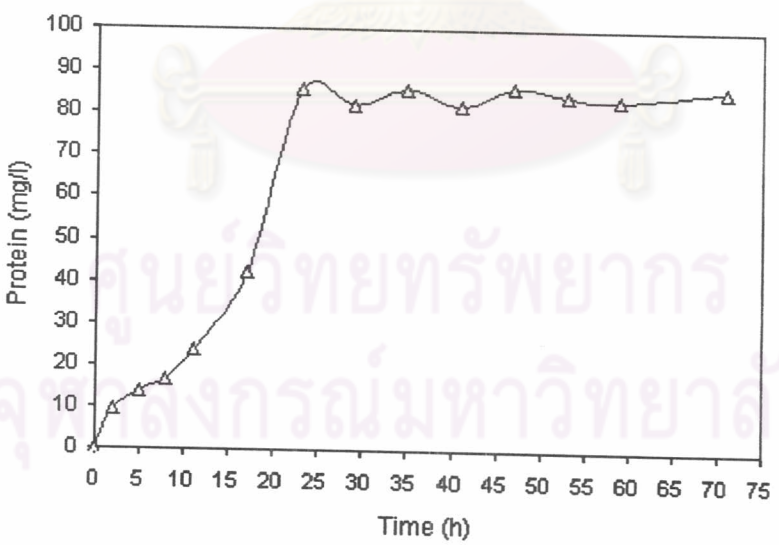

Figure 4.3: Time-course of protein in conventional alkaline protease fermentation. Conditions: pH 7 and at 37 °C and at 250 rpm agitation,

From figure 4.1, concentration of glucose was rapidly reduced after 2 hours and was slowly reduced after 8 hours of inoculation. The consumption rate in this

time interval was 0.763 g l^{-1} h^{-1}. This is in accordance with the cell concentration in figure 4.2, which increased rapidly during the same time interval and growth rate was 5.782E-4 g l^{-1} h^{-1}. The alkaline protease production was delayed for 6 hours compared with that of the cell production. Alkaline protease production began increasing rapidly when cells were in stationary phase, therefore, it could be concluded that alkaline protease is a mixed-growth associate product (figure 4.2). After 71 hours of fermentation the specific purity of alkaline protease that based on total amount of protein was 12.83 unit/ mg protein, the time-course of protein production was shifted for 6 hours from enzyme profile (figure 4.3).

From figure 4.2 we can clearly see that after 17 hours of inoculation alkaline protease was not produced anymore, this result was according to the very small amount of glucose left (figure 4.1).

4.2 PEG 1000/ potassium phosphate aqueous two-phase system

4.2.1 Phase diagram

Figure 4.4 demonstrates phase diagram of PEG 1000/ potassium phosphate ATPs that was constructed at system pH 7and room temperature (30°C ± 2°C). The left-handed side of the binodal curve demonstrates homogeneous phase system while the right-handed side designated aqueous two-phase system. Points A, B, C...specify system compositions which extractive fermentations were carried out, while compositions at the end of each tie-line define compositions of each phase. The PEG rich phase is lighter and layers on top of the phosphate-rich phase.

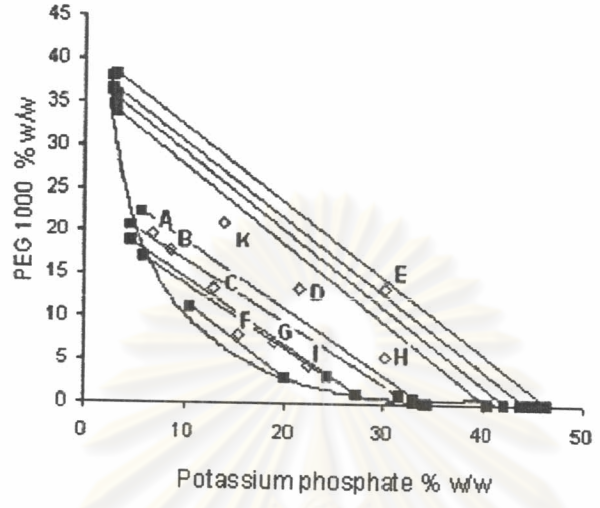

Figure 4.4: Rectangular phase diagram of PEG 1000/ potassium phosphate systems at system pH 7 and room temperature (30°C± 2°C). Points A, B, C...indicated system compositions for which fermentation in ATPs were conducted.

4.2.2 Extractive fermentation:

Based on a previous study (Chouyok, 2001), the best system composition for extraction of alkaline protease was mixture of 21 % w/w PEG 1000 and 14.5 %w/w of potassium phosphate (point K figure 4.4)). This composition was selected as a primary choice for extractive fermentation. Results of alkaline protease production in aqueous two-phase system are represented in Figure 4.5. It was obviously noticed that the substrate glucose was not utilized in ATPs, as a result, alkaline protease production was not observed. Therefore, it could be concluded that under this optimal conditions for alkaline protease extraction, cell growth and product formation did not occur in ATPs for the PEG 1000/ potassium phosphate system.

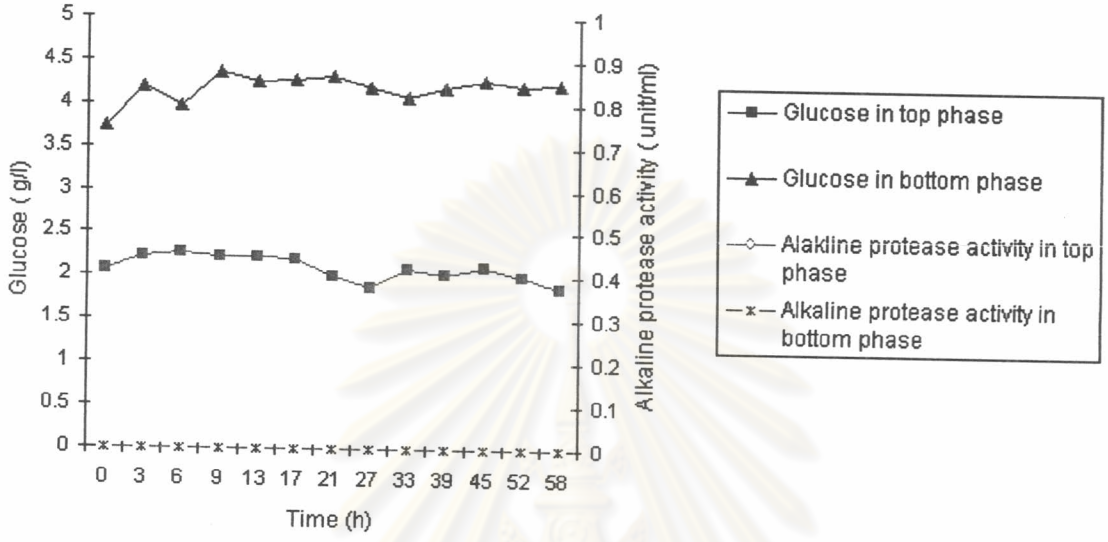

Figure 4.5: Time-course of alkaline protease production and glucose concentration ATPs of 21 %w/w PEG 1000 and 14.5 % w/w of potassium phosphate in shake flasks at 250 rpm agitation, pH 7 and room temperature (30°C ± 2°C).

In order to search for better PEG 1000/ potassium phosphate ATPs for extractive alkaline protease production, wide range of compositions of these particular ATPs were investigated. Table 4.1 elaborates these system compositions and their corresponding phase volume ratios

Thoroughly explored almost throughout the whole two-phase area of the PEG 1000 / potassium phosphate diagram (figure 4.4), alkaline protease extractive fermentation was not accomplished in all the systems ranging from PEG 1000 concentration of 4.45-21 %w/w, potassium phosphate concentration of 6.88-30.1 %w/w, and volume ratios of 0.2-5.4.

Therefore, we can conclude that PEG 1000/ potassium phosphate system was unsuitable for extractive fermentation of *Bacillus subtilis* TISTR 25 to produce alkaline protease in pH 7 and at ambient temperature.

Table 4.1: Compositions chosen for alkaline protease extractive fermentations (also see figure 4.4).

Points	PEG 1000 (%w/w)	Potassium Phosphate (%w/w)	Volume ratio $R_v = V_T / V_B$
K	21	14.5	1.4
A	19.58	6.88	5.4
B	17.8	8.6	4.8
C	13.35	12.9	1.3
D	13.35	21.5	0.8
E	13.35	30.1	0.4
F	8.01	15.48	1
G	7.12	18.92	0.5
H	5.34	33.1	0.2
I	4.45	22.36	0.3

4.3 PEG X (4000, 6000 and 10000)/ phosphate aqueous two-phase systems.

4.3.1 Phase diagram:

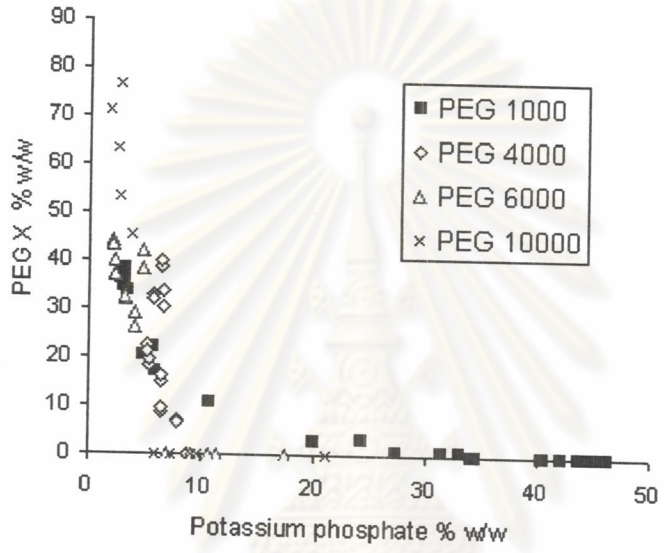

Figure 4.6: Rectangular phase diagram for varied molecular weights of PEG/ potassium phosphate systems at pH 7, and room temperature (30°C± 2°C).

From the results shown in figure 4.6, binodal curves of various PEG systems (PEG X/potassium phosphate/fermentation medium) did not show significant variations. However, that of ATPs with PEG1000 was found further off from the origin, in addition, its bottom phase contained measurable amounts of PEG while only trace amounts were found in the others. This was corresponding to Boris (1995), who reported that with the same type of polymer, the higher molecular weight results in shifting of binodal curve towards the original point [(0,0) co-ordinate]. However, the slope of the binodal curve was found to be altered. PEG 1000/ potassium phosphate binodal curve made a triangular with axes of co-ordinate, but in the upper part of phase diagram of PEG X (4000, 6000, and 10000)/ potassium phosphate was paralleled with vertical axis while lower part seems lie on the horizontal axis. Therefore, it could be pointed out that phase diagram ATPs with molecular weights 4000 and higher were very sensitive to change in PEG concentration. However, with different polymer type, more dramatic change in phase separation would be observed than in case the same polymer type but different molecular weight (Albertsion, 1986).

4.3.2 General system characteristics:

Figure 4.7 illustrates visual characteristics of PEG 4000/ potassium phosphate/ reaction media ATPs of varied system compositions and volume ratios. Both phases were of yellowish color, however, darker color was noticed in PEG-rich top phase. This was probably due to higher partition of yeast extract to the top phase than to the bottom potassium-rich phase. Therefore, we could anticipate that some impurities from fermentation broth would interfere partitioning and lower the specific activity of alkaline protease in the top PEG-rich phase. These same visual characteristics were observed in all PEG X / potassium phosphate ATPs.

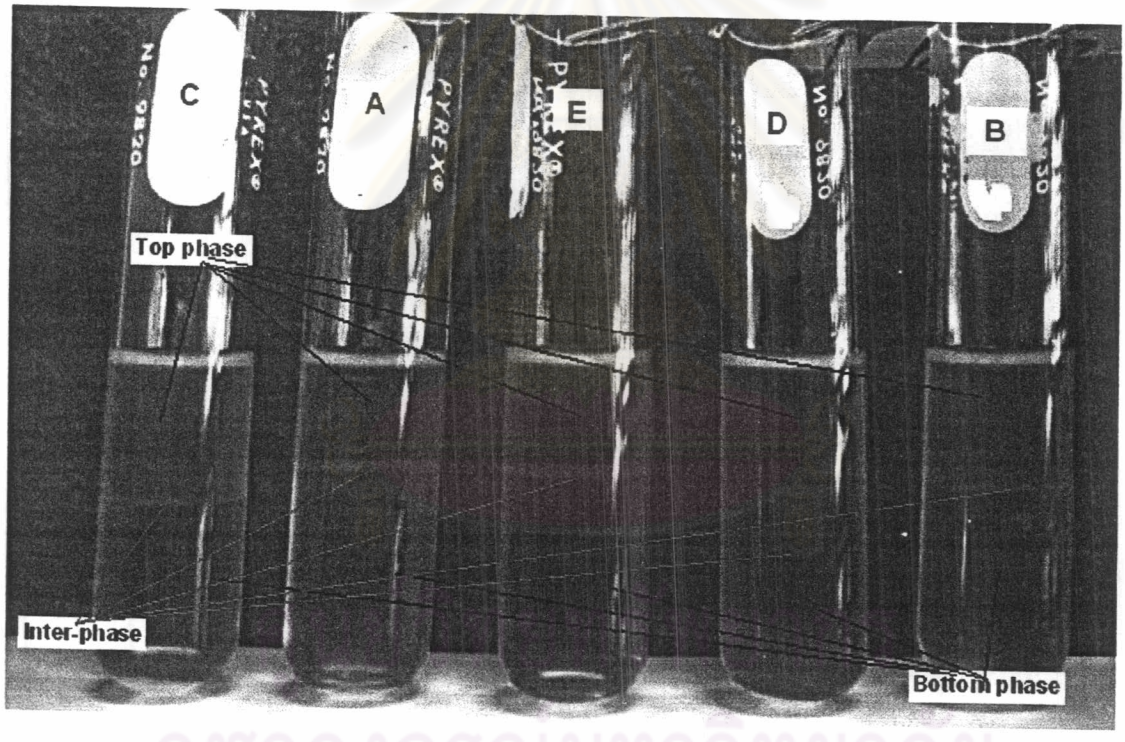

Figure 4.7: ATPs of PEG 4000/ potassium phosphate at pH 7 and room temperature (30°C ± 2°C). Points A, B, C, D, E refers to system composition which extractive fermentation was carried out as given in table 4.3 and 4.4 and figure 4.20.

During fermentation, for all ATPs of various PEG molecular weights, cells were mostly found in the bottom and inter-phase (Figure 4.8). With increase in fermentation time, the bottom phase becomes more and more viscous with gel-like characteristic. This caused in homogeneity in potassium phosphate-rich

bottom phase. Thus high measurement errors on cell, alkaline protease, and protein concentrations were observed. As a result, measurements of these compounds were only done in the PEG-rich top phase. Since most of the cells were sited in the bottom phase, gel-like characteristic observed after some fermentation time may be generated by some cells excreted biomolecules whether from dead or living cells. To clarify this point, more experiments are needed.

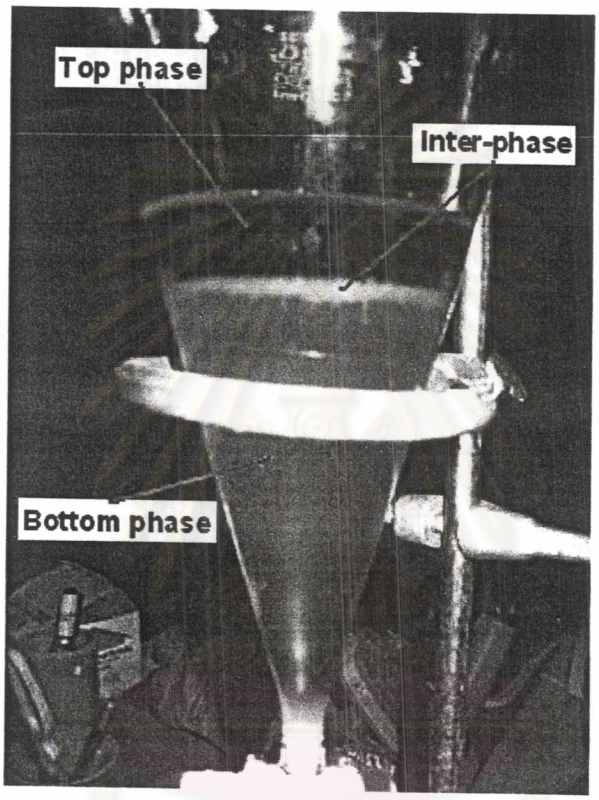

Figure 4.8: Visual characteristic of ATPs obtained by 12 % w/w PEG 10000 and 18 % w/w potassium phosphate after 71 hours of inoculation.

4.3.3 Alkaline protease extractive fermentation in ATPs of varied PEG molecular weights:

To explore for other ATPs which might give better results for alkaline protease production than PEG 1000/potassium phosphate ATPs, PEG X (X = 4000, 6000, and 10000)/ potassium phosphate systems were investigated. Hotha (Hotha et al, 1997) reported alkaline protease production by using *B.*

thuringensis H14 with PEG molecular weights of 4000, 6000 and 9000 respectively. The compositions were as the following:

Table 4.2: ATPs compositions for alkaline protease extractive fermentation by *B. thuringensis* H14 *.

Systems	PEG (%w/w)	Potassium phosphate (% w/w)	Volume ratio
PEG 4000/ phosphate	13.6	13.75	1.08
PEG 6000/ phosphate	14.3	11.25	1.13
PEG9000/ phosphate	14.6	9.42	1.1

* Hotha et al, 1997,

In order to select suitable PEG molecular weight for extractive fermentation, a series of system with the same compositions (12.46 % w/w of PEG X and 9.89 % w/w of potassium phosphate) was setup for PEG 4000, 6000 and 10000, respectively. This particular system compositions were obtained by averaging Hotha (1997)'s compositions as given in Table 4.2. The volume ratios determined in our study were 0.75, 0.74 and 0.71 for PEG 4000, 6000 and 10000, respectively. This could be considered as very similar phase volume ratio values. Therefore, effects due to varied phase volume ratios could be ruled out and the different results that might occur could be due only to different molecular weights of PEG.

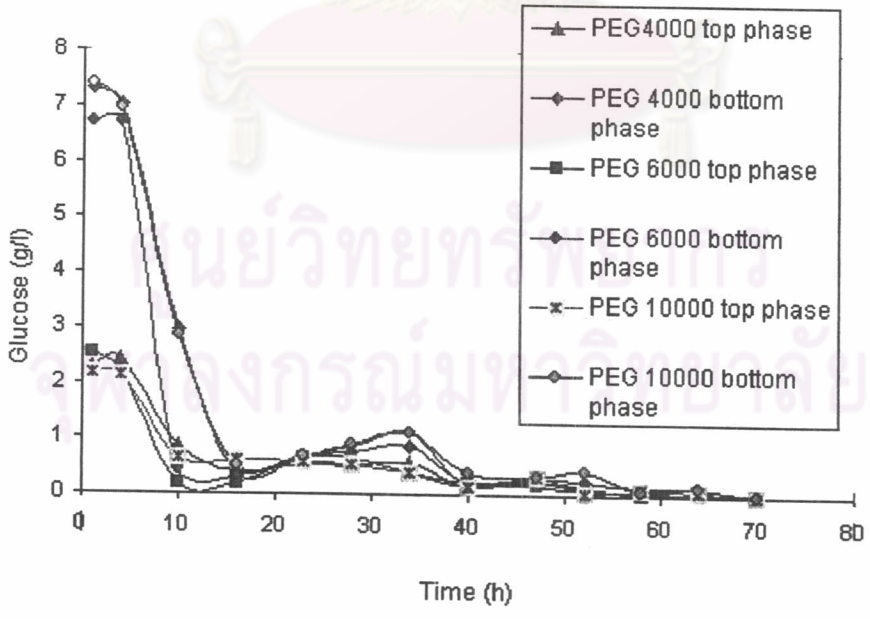

Figure 4.9: Time-course of glucose concentration in ATPs obtained by 12.46 %w/w of PEG X (4000, 6000, and 10000) and 9.89 %w/w of potassium phosphate system. Conditions: pH 7, 37° C and 250 rpm of stirrer.

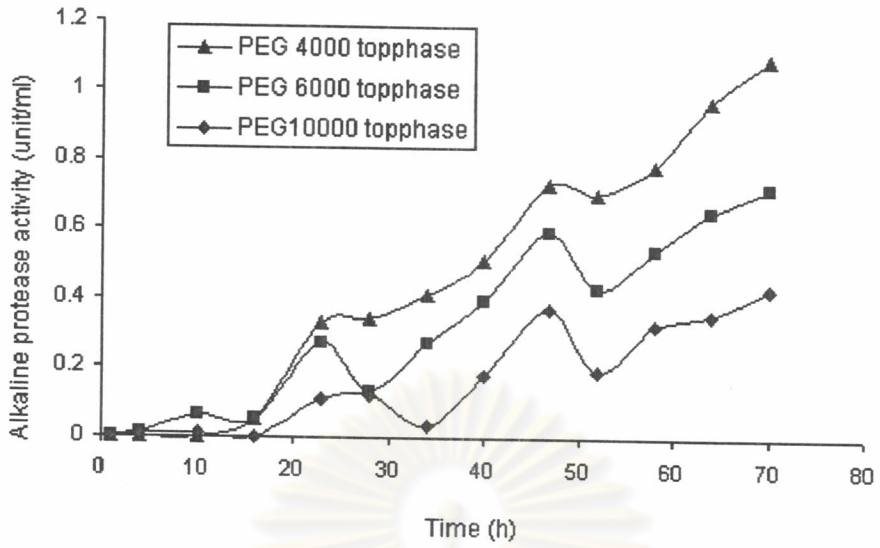

Figure 4.10: Time-course of alkaline protease activity in top phase of ATPs obtained by 12.46 %w/w of PEG X (4000, 6000, and 10000) and 9.89 %w/w of potassium phosphate system. Conditions: pH 7, 37° C and 250 rpm of stirrer

Figure 4.9 demonstrates time-course glucose concentrations both in top PEG X phase and bottom potassium phosphate phase. Considering at zero fermentation time, partition coefficients of glucose were determined at 0.313, 0.378 and 0.295 for PEG 4000, 6000, and 10000 ATPs, respectively. This clearly demonstrated that glucose was more concentrated in the bottom phase and readily available for cells which resided mainly in this same potassium phosphate-rich phase. Concentrations of glucose in the bottom phase were 1.217, 1.115, and 1.23 times higher than that in conventional fermentation solution. Glucose consumption rate on the time interval of [4h; 10h] after inoculation in the bottom phase were 0.678 g/l/h, 1.050 g/l/h and 0.745 g/l/h in ATPs of PEG X (4000, 6000 and 10000)/ potassium phosphate, respectively. After 4 hours of inoculation, concentration of glucose in the top phase where no cell was resided was also decreased. These can be easily explained by glucose mass transfer from top phase to bottom phase.

During the first 17 hours of fermentation, very low alkaline protease production rates were observed. This may be due to low cell concentration in this beginning period that cause slow alkaline protease accumulation. In addition, change in system environment from normal fermentation broth in the inoculum to ATPs in the fermentation medium may cause longer lay phase thus later enzyme production (Andersson, 1985).

The results shown in Figure 4.10 also indicated that with increasing polymer molecular weight decreasing alkaline protease production rate was detected. After 71 hours the total amount of alkaline protease in PEG 4000 system was 0.61 unit/ml, which was clearly higher than that of PEG 6000 (0.345 unit/ml) and PEG 10000 (0.284 unit/ml). It could be concluded that higher PEG molecular weight caused lower alkaline protease production rate. These results correspond to Hotha's (1997) who reported that total alkaline protease

production was 2.38, 2.61 and 2.8 times higher than that of homogeneous fermentation when molecular weights of PEG were 9000, 6000 and 4000 were applied, respectively. Therefore, we decided to choose PEG 4000 as an optimal molecular weight for further extractive fermentations.

The reason that PEG molecular weights 4000 and higher were more suitable for cell growth than PEG 1000 may due to high stress caused by high concentration of polymer and potassium phosphate in the bottom phase that required to make ATPs for PEG 1000 compared with ATPs of PEG X (4000, 6000, and 10000) and present of PEG 1000 in the bottom phase as shown in Figure 4.6. This phenomenon according to Kuboi (1995), who reported that low-molecular-weight PEG has been found to be inhibitory to cell growth, possibly due to their interaction with the cell walls of microorganisms (Honda et al, 1981).

To deeper understand about the effect of molecular weight on cell growth and morphology, the shape of cells during extractive fermentation was observed by light microscope and pictures were taken by digital camera Canon Powershot S50 compared between control system and in the ATPs. The same composition as 10.68 % w/w of PEG X (1000; 4000; 6000; and 10,000) and 15.48 % w/w of potassium phosphate was selected which is the point closed to plait point of binodal curve for PEG 1000.

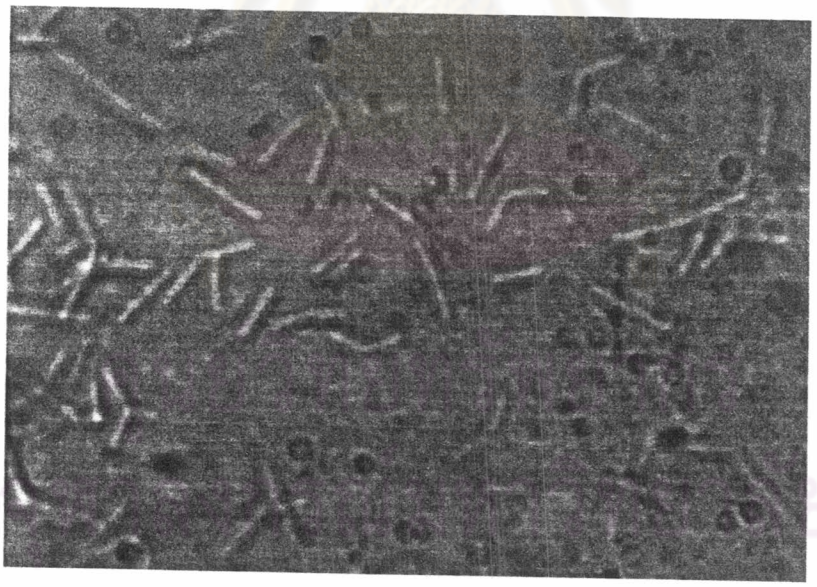

Figure 4.11: Shape of *Bacillus subtilis* TISTR 25 in conventional fermentation at pH 7 and 30°C ± 2°C after 17 hours of fermentation.

Figure 4.12: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 30°C± 2°C at the 17 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 4000 and 15.48 % w/w of potassium phosphate.

Figure 4.13: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 30°C± 2°C at the 48 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 1000 and 15.48 % w/w of potassium phosphate.

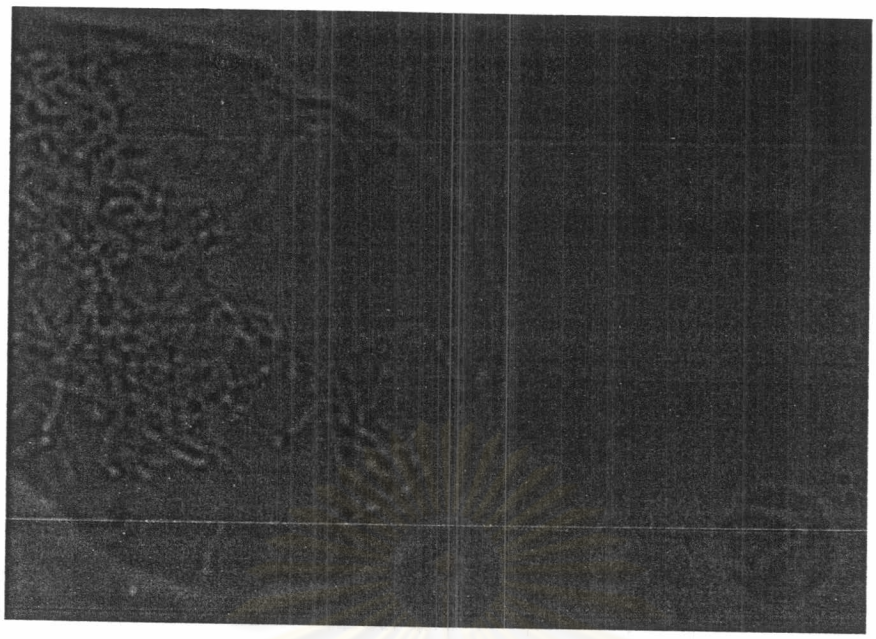

Figure 4.14: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 30°C± 2°C at the 48 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 4000 and 15.48 % w/w of potassium phosphate.

Figure 4.15: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 30°C± 2°C at the 48 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 6000 and 15.48 % w/w of potassium phosphate.

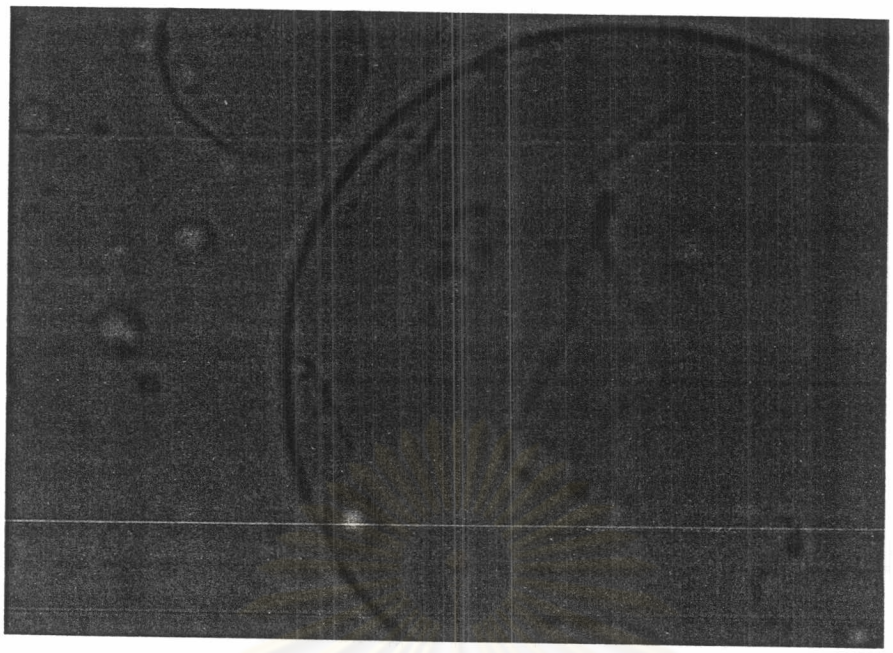

Figure 4.16: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 30°C± 2°C at the 48 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 10000 and 15.48 % w/wf potassium phosphate.

In the normal fermentation, the shape of *Bacillus subtilis* TISTR 25 was rod type (figure 4.11). Ater 17 hours of inoculation in ATPs, under high stress of polymer and potassium phosphate concentration, it was broken to a lot of small piece and made spore coat for self-protection (Peter et al, 1984). This phenomenon was occurred in all PEG molecular weights of ATPs (figure 4.12 – ATPs with PEG 4000). After inoculation for 48 hours the cell was grown in ATPs of PEG 4000, 6000, 10000 (figure 4.14, 4.15 and 4.16) but not in ATPs of PEG 1000 (figure 4.13). In these figures, the circle phase represents bottom phase and the continuous represents top phase. More interestingly, under high stress the originally rod shaped cells were found elongated in all ATPs (figure 4.14, 4.15, and 4.16).

For longer fermentation time, when cell went to dead phase, cells were broken into small pieces and aggregated at inter-phase and in the bottom phase, a little of it was in the top phase (figure 4.17, 4.18, and 4.19). This phenomenon caused difficulty in cell separation when we tried to separate cell from aqueous solution as mentioned in part 4.3.2.

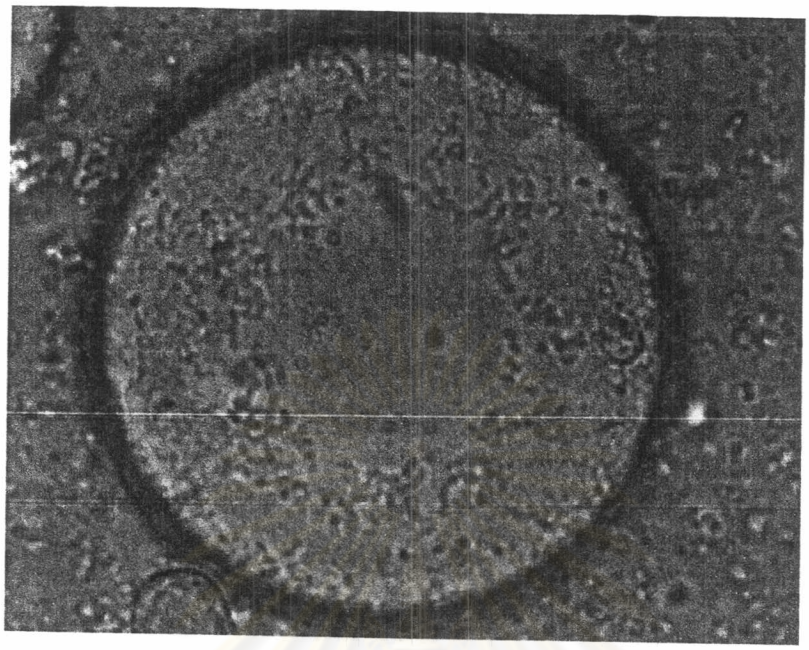

Figure 4.17: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 37^{0}C at the 71 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 4000 and 15.48 % w/w of potassium phosphate.

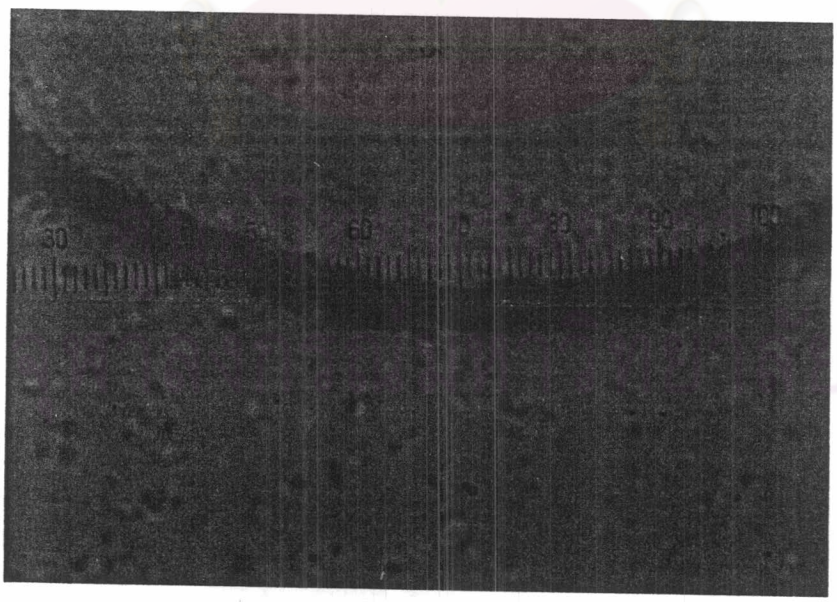

Figure 4.18: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 37^{0}C at the 71 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 6000 and 15.48 % w/w of potassium phosphate.

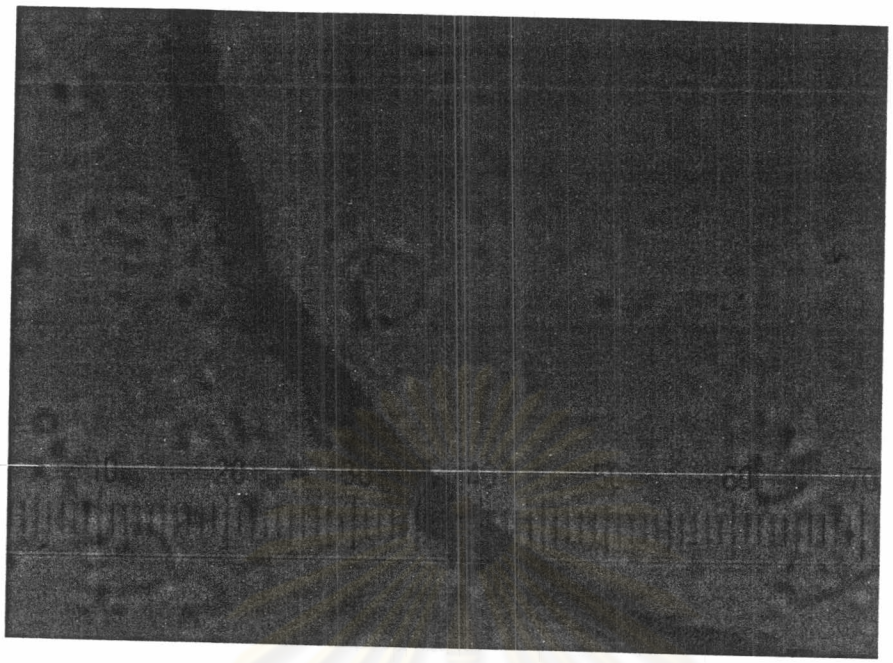

Figure 4.19: Shape of *Bacillus subtilis* TISTR 25 at pH 7 and 37°C at the 71 hours of fermentation in bottom phase of ATPs obtained by 10.68 % w/w of PEG 10000 and 15.48 % w/w of potassium phosphate.

4.4 PEG 4000/ potassium phosphate aqueous two-phase systems.

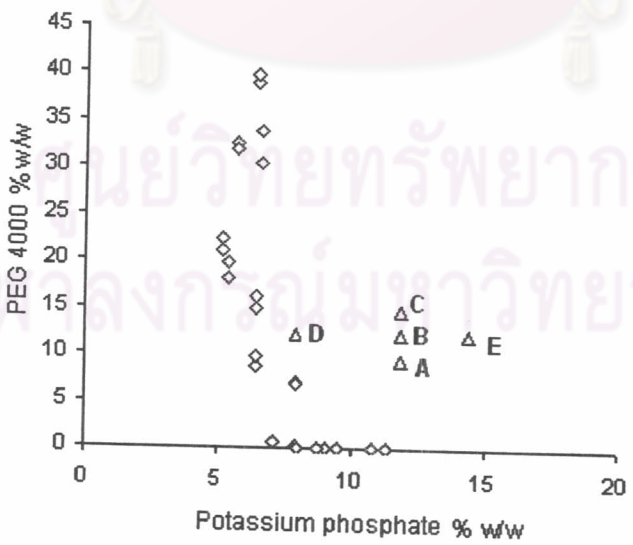

Figure 4.20: Binodal curve of PEG 4000/ potassium phosphate ATPs at system pH7, room temperature (30°C ± 2°C). Points A, B, C, D, and E indicate system compositions which extractive fermentation were carried out.

Since we discovered that PEG 4000 gave best results for alkaline protease extractive fermentation, further experiments were carried out to investigate effects of PEG 4000 and potassium phosphate concentration on alkaline protease production. These system compositions were selected based on Hotha's report which designated point B (13.6 %w/w pf PEG 4000, 13.75 %w/w of potassium phosphate) as best composition for alkaline protease extractive fermentation by *B. thuringensis* H14 (Hotha et al, 1997).

4.4.1 Effect of PEG 4000 concentration on extractive fermentation:

Table 4.3: ATPs compositions of PEG 4000/ potassium phosphate systems: to investigate effect of PEG 4000 concentration on extractive fermentation.

Points	PEG 4000 (% w/w)	Potassium phosphate (%w/w)	Volume ratio
A	10.6	13.75	0.379
B	13.6	13.75	0.645
C	16.6	13.75	0.8

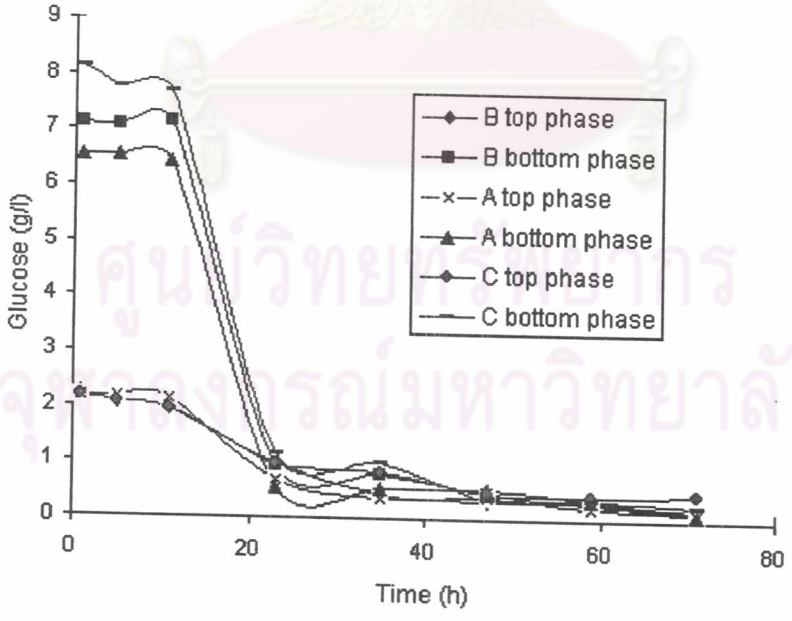

Figure 4.21: Glucose concentration in ATPs with compositions as given in Table 4.3. Fermentation conditions: pH 7, 37° C and 250 rpm of stirrer.

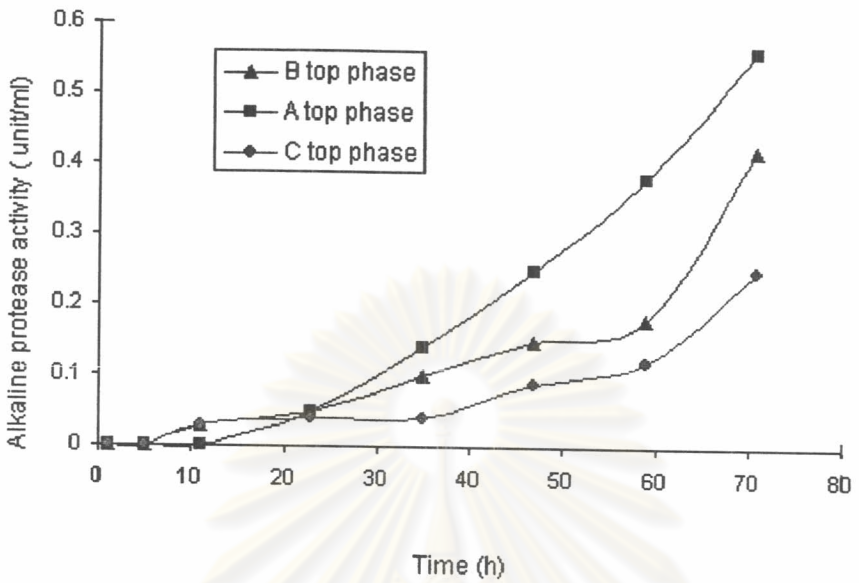

Figure 4.22: Alkaline protease activity in the top phase of ATPs obtained by compositions given in Table 4.3 in fermentor at pH 7, 37° C and 250 rpm of stirrer.

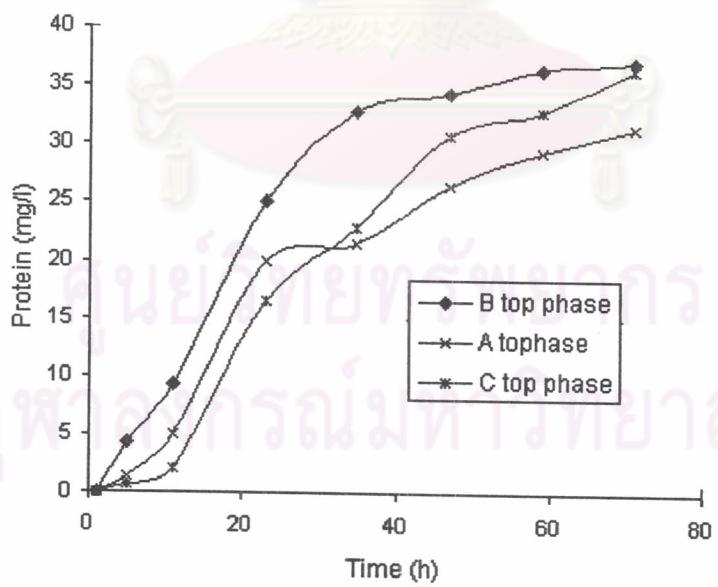

Figure 4.23: Protein concentration in the top phase of ATPs obtained by compositions given in Table 4.3 in fermentor at pH 7, 37° C and 250 rpm of stirrer.

From Table 4.3, it is obviously noticed that by fixing potassium phosphate concentration at 13.75 %w/w and varied PEG 4000 concentration, phase volume ratio and phase composition were changed. With concentration of PEG 4000 were 10.6%w/w, 13.6%wt and 16.6%w/w the volume ratio was increased from 0.379, to 0.695 and to 0.8, respectively. This can be explained by a very high tie-lie slope of binodal curve of PEG 4000. Varied system compositions also altered partition coefficients of glucose 0.332, to 0.312 and to 0.265 for ATPs obtained by points A, B and C, respectively with a significant change in the bottom phase when that in the top phase was quite the same (figure 4.21). This result was according to Zaslavsky (1995) who reported that when total polymer concentration is increased the solute partitioning becomes more one-sided. The consumption rate of glucose was 0.489 g l^{-1} h^{-1}, 0.514 g l^{-1} h^{-1}, and 0.548 g l^{-1} h^{-1}after 11 hours and rapidly reduced after 23 hours of inoculation. That was 1.56, 1.484 and 1.392 times lower than that in conventional fermentation; this may be explained by inhibition of PEG in ATPs.The alkaline protease activity in the top phase was also decreased as 0.56 unit/ml, 0.42 unit/ml and 0.25 unit/ml after 71 hours (figure 4.22). The concentration of protein in the top phase also did not show the change by PEG 4000 concentration (figure 4.23). Even those, after 71 hours of fermentation the specific purity of alkaline protease in the top phase were 17.818 unit/mg protein, 11.307 unit/mg protein and 6.863 unit/mg protein for ATPs obtained by point A, B and C (Table 4.3), respectively.

The purification factor is defined as following:

Purification factor = (Specific purity in the top phase)/ (Specific purity in the conventional fermentation)

Purification factors were 1.389, 0.880 and 0.536 times compared with that in conventional fermentation with ATPs obtained by points A, B and C, respectively. After 71 hours of inoculation, the production rate bases on amount of alkaline protease on the top phase were 7.89E-3 units ml^{-1} h^{-1}, 5.9E-3 units ml^{-1} h^{-1} and 3.5E-3 units ml^{-1} h^{-1} with ATPs obtained by points A, B and C, respectively.

4.4.2 Effect of potassium phosphate concentration on extractive fermentation:

Table 4.4: ATPs compositions of PEG 4000/ potassium phosphate systems: to investigate effect of potassium phosphate concentration on extractive fermentation.

Points	PEG 4000 (% w/w)	Potassium phosphate (%w/w)	Volume ratio
D	13.6	9.25	1.155
B	13.6	13.75	0.645
E	13.6	16.75	0.538

The change of concentration of potassium phosphate caused the change in phase volume ratio and phase composition. With an opposite effect from PEG concentration, the higher concentration of phosphate, the lower phase volume ratio (Table 4.4). This change also caused decrease in glucose partition coefficient which was determined at 0.42, 0.33 and 0.25 corresponding to potassium phosphate concentration of 9.27 %w/w, 13.75 %w/w and 16.75 %w/w, respectively, with fixed PEG 4000 concentration at 13.6 %wt. Concentrations of glucose in the bottom phase are quite similar, while in the top phase are significantly different. This opposite to results from Figure 4.19 may be due to high concentration of phosphate in ATPs. The maximum consumption rate of glucose was defined as 0.352 g l^{-1} h^{-1}, 0.514 l^{-1} h^{-1} and 0.545 l^{-1} h^{-1} with ATPs obtained by points D, B and E as given in Table 4.4, respectively. That was 2.168, 1.484 and 1.4 times lower than that in conventional fermentation.

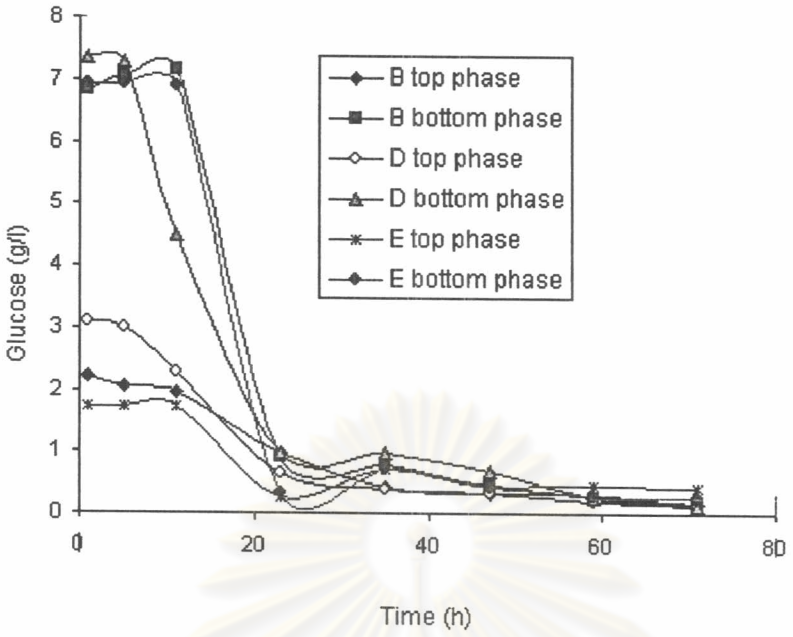

Figure 4.24: Reducing glucose of ATPs obtained by compositions given in Table 4.4 in fermentor at pH 7, 37° C and 250 rpm of stirrer

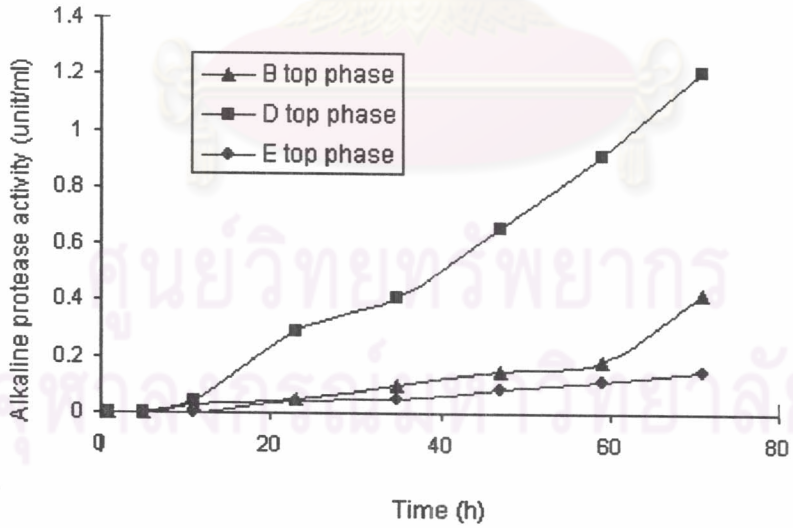

Figure 4.25: Alkaline protease activity in the top phase of ATPs obtained by compositions given in Table 4.4 in fermentor at pH 7, 37° C and 250 rpm of stirrer.

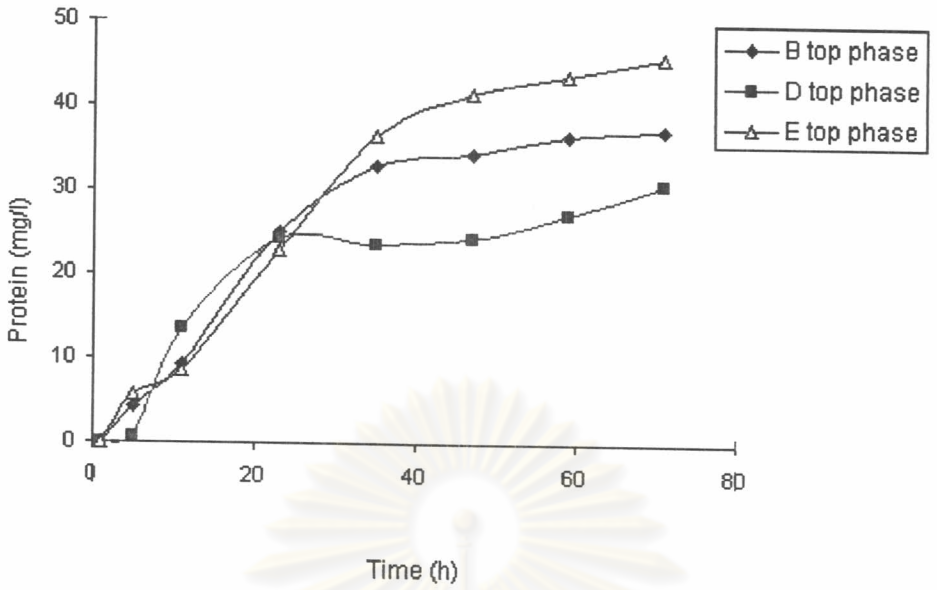

Figure 4.26: Protein in the top phase of ATPs obtained by compositions given in Table 4.4 in fermentor at pH 7, 37° C and 250 rpm of stirrer

After 71 hours of extractive fermentation, alkaline protease activity in the top phase was 1.21unit/ml, 0.42 unit/ml and 0.15 unit/ml with the increasing concentration of phosphate as points D, B and E (Figure 4.25). Figure 4.26 indicated that high phosphate concentration caused low concentration of protein in the top phase. This also improved the purity of extracted alkaline protease, but also reduced enzyme production. However, the specific purity of alkaline protease in the top phase was decreased from 39.395 unit/mg protein to 11.307 unit/mg protein and 3.28 unit/mg protein with increasing of potassium phosphate as points D, B and E given in Table 4.4, respectively. The production rate of alkaline protease baseed on amount of enzyme in the top phase was defined as 9.13E-3 units ml^{-1} h^{-1}, 2.32E-3 units ml^{-1} h^{-1} and 0.74E-3 units ml^{-1} h^{-1}. Purification factors after 71 hours of inoculation were defined as 3.07, 0.881 and 0.256 with the increasing concentration of phosphate as points D, B and E respectively. However, current knowledge about effect of salts on the interactions of biopolymers with aqueous medium, which was an important factor in ATPs, is still very limited. (Boris, 1995).

From figure 4.20, 4.22 and 4.25 we can conclude that the closer the tie-lie to the plait point of binodal curve, the better conditions for extractive fermentation.

4.4.3 Effect of volume ratio on extractive fermentation:

For all molecular weight of PEG, the shorter tie-line, the higher alkaline protease was produced (Hotha et al, 1997). Therefore, effects of volume ratio on extractive fermentation were studied in tie-line as nearest to the plait point of PEG 4000 binodal curve as possible. The systems are as the following:

Table 4.5: ATPs compositions of PEG 4000/ potassium phosphate systems: to investigate effect of phase volume ratio on extractive fermentation.

Points	PEG 4000 %w/w	Potassium phosphate %w/w	Volume ratio	Alkaline protease partition coefficient
A1	15.68	7.18	2.22	5.67
A2	11.89	8.23	1.34	7. 18
A3	9.75	8.78	0.91	26.75
A4	10.2	9.36	0.54	1.26

Table 4.5 illustrates that, along a tie-line, partition coefficient of alkaline protease decreased if the phase volume ratio shifted away from 1.0. Point A3 gave maximum alkaline protease partition coefficient (26.075) at phase volume ratio of 0.91. These ATPs is a promising system for alkaline protease extraction. The similar result was reported by Srinivas (1997) that at the phase volume ratio 1.08 the highest alkaline protease partition coefficient was found out as 4.07 (Table 4.6). With the phase volume ratio of 0.54, partition coefficient of alkaline protease was found smallest (1.26).

Table 4.6: Effect of volume ratio on alkaline protease partitioning in PEG 4000 – phosphate-water system [*].

Points	PEG 4000 %w/w	Potassium Phosphate %w/w	Volume ratio	Alkaline protease partition coefficient
1	15.3	13.10	6.69	1.849
2	14.5	13.38	2.85	2. 647
3	13.6	13.75	1.08	4.700
4	12.6	14.13	0.47	4.450
5	11.7	14.30	0.32	4.451

[*] Hotha et al (1997).

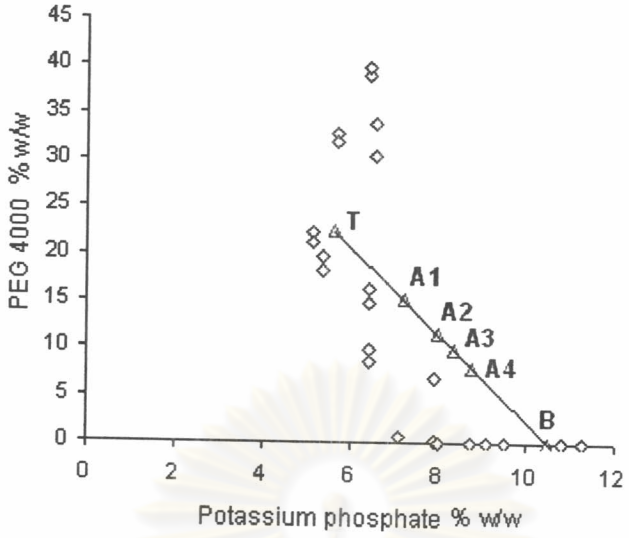

Figure 4.27: ATPs compositions of PEG 4000/ potassium phosphate systems: to investigate effect of phase volume ratio on extractive fermentation. The top composition was 5.64 %wt of potassium phosphate and 22.43 %w/w of PEG 4000 (Point T) when the bottom phase was 10.51 %w/w of phosphate without present of PEG 4000 (point B). Phase conditions were pH 7 and room temperature.

4.3.3.2 Alkaline protease production

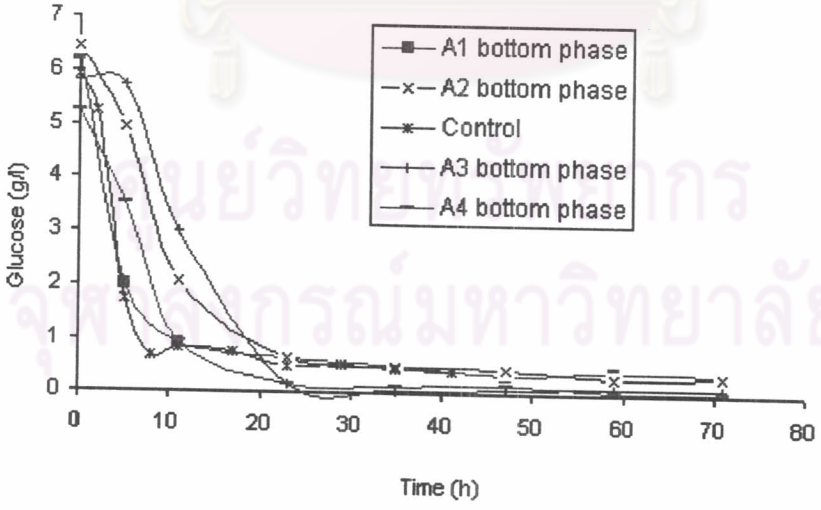

Figure 4.28: Time-course of glucose in the bottom phase of ATPs obtained by compositions given in Table 4.5. Conditions: pH 7, 37° C and 250 rpm of stirrer.

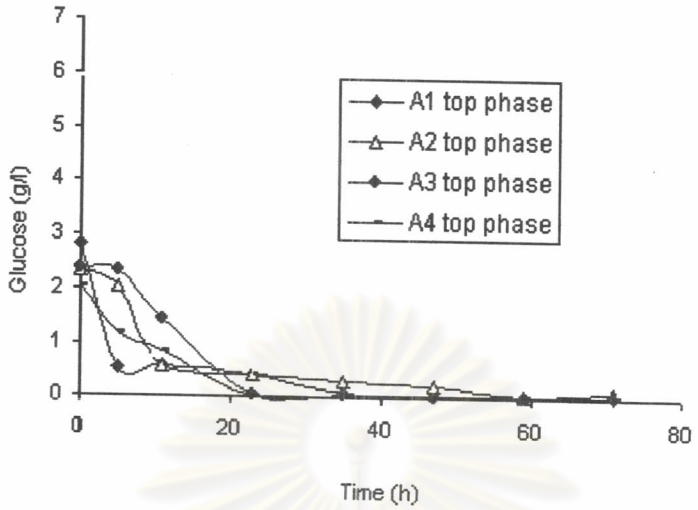

Figure 4.29: Time-course of glucose in the top phase of ATPs obtained by compositions given in Table 4.5. Conditions: pH 7, 37° C and 250 rpm of stirrer.

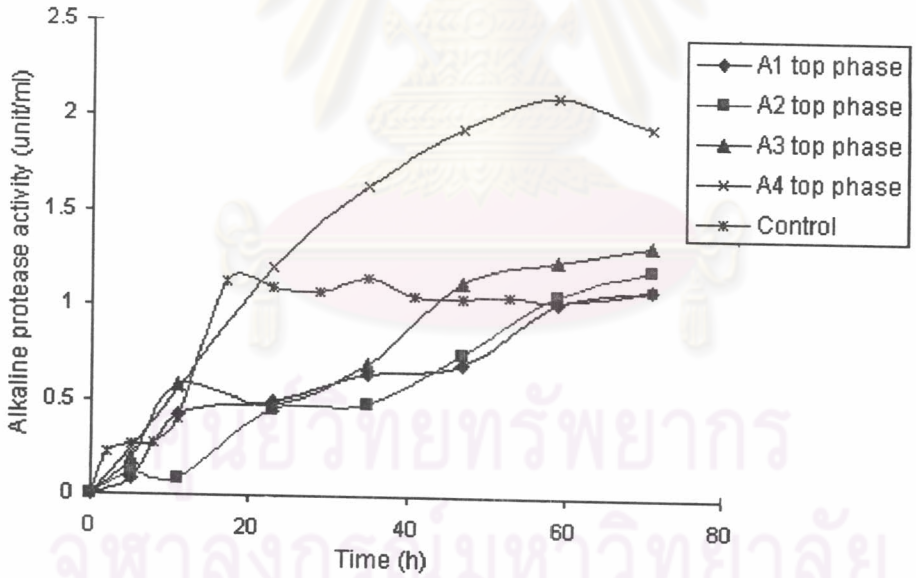

Figure 4.30: Time-course of alkaline protease activity in the top phase of ATPs obtained by compositions given in Table 4.5. Conditions: at pH 7, 37° C and 250 rpm of stirrer.

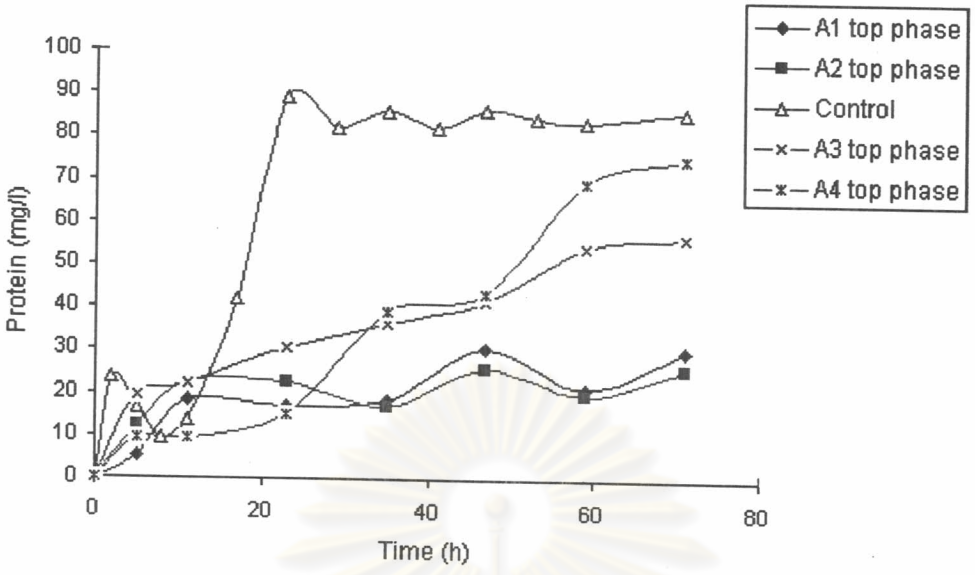

Figure 4.31: Time-course of protein in the top phase of ATPs obtained by compositions given in Table 4.5. Conditions: at pH 7, 37° C and 250 rpm of stirrer

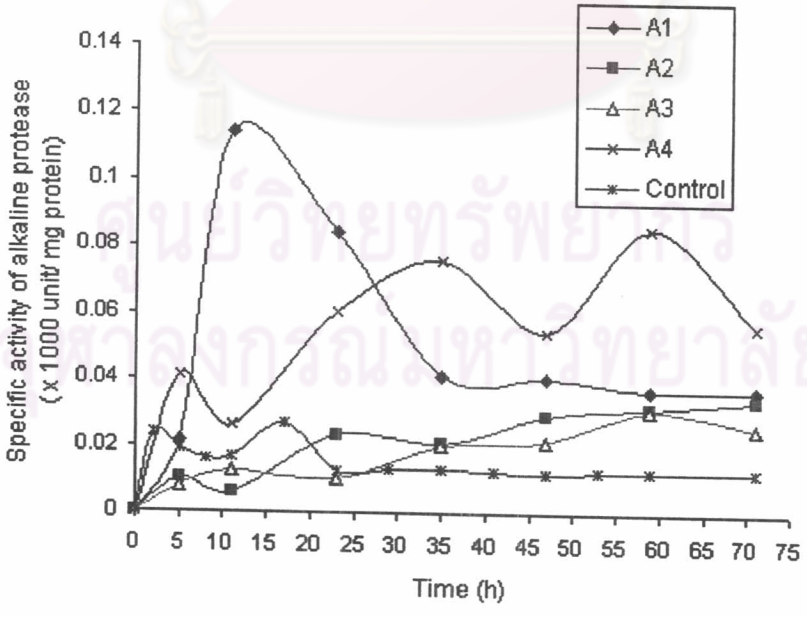

Figure 4.32: Time-course of specific activity of alkaline protease in the top phase of ATPs obtained by compositions given in Table 4.5, Conditions: at pH 7, 37° C and 250 rpm of stirrer

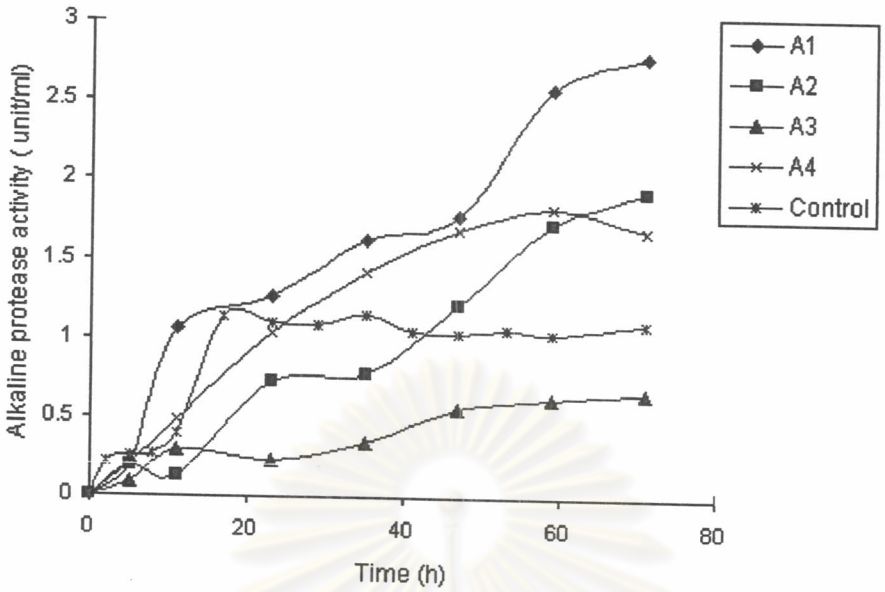

Figure 4.33: Time-course of total alkaline protease activity in ATPs obtained by compositions given in Table 4.5. Conditions: at pH 7, 37° C and 250 rpm of stirrer.

Figure 4.28 and 4.29 show that glucose concentrations in the top phase in all ATPs were not much different from that in conventional fermentation.

With decreasing the volume ratio along a tie-line, the alkaline protease in the top phase increased. Figure 4.30 illustrates that after 71 hours of extractive fermentation, alkaline protease activity in the top phase was 1.95 unit/ml, 1.33 unit/ml, 1.2 unit/ml and 1.11 unit/ml with the volume ratio was 0.54, 0.91, 1.34 and 2.22 respectively. A change of volume ratio also resulted in changing of protein concentration in the top phase. The higher volume ratio resulted in the lower protein concentration in the top phase (figure 4.31). However, the purity of alkaline protease in the top phase in all cases were still significantly higher than that of conventional fermentation (figure 4.32). It can be concluded by purification factors, which were 2.88, 2.66, 2.0 and 4.387. The total alkaline protease in ATPs did not follow the rule because of the dependence of that on volume ratio and alkaline protease partition coefficient (figure 4.33). That was 2.517, 1.755, 1.535 and 0.6 times compared with conventional fermentation. Phase volume ratio also effected viscosity of ATPs , the higher phase volume ratio, the higher viscosity of system. This was causal reducing oxygen transformation and dissolved oxygen.

The top phase and normal fermented broth without cell were kept at 4 ^{0}C for 3 weeks, and then one week at ambient temperature. For the all phase volume ratios, the stability of alkaline protease in the top phase were not changed during storage time. That is also observed in the fermented broth (results not shown).

4.3.3.1 The shape of cell

After inoculums 5 hours, 24 hours and 48 hours the shape of cell was observed by electron scanning as following:

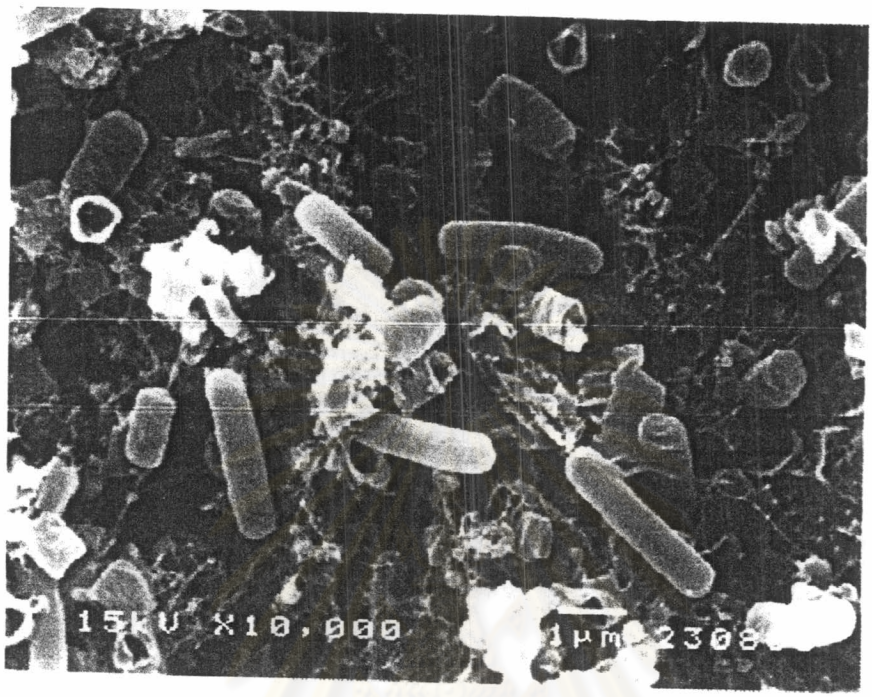

Figure 4.34: The shape of cell in ATPs obtained by point A1 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 5 hours of fermentation.

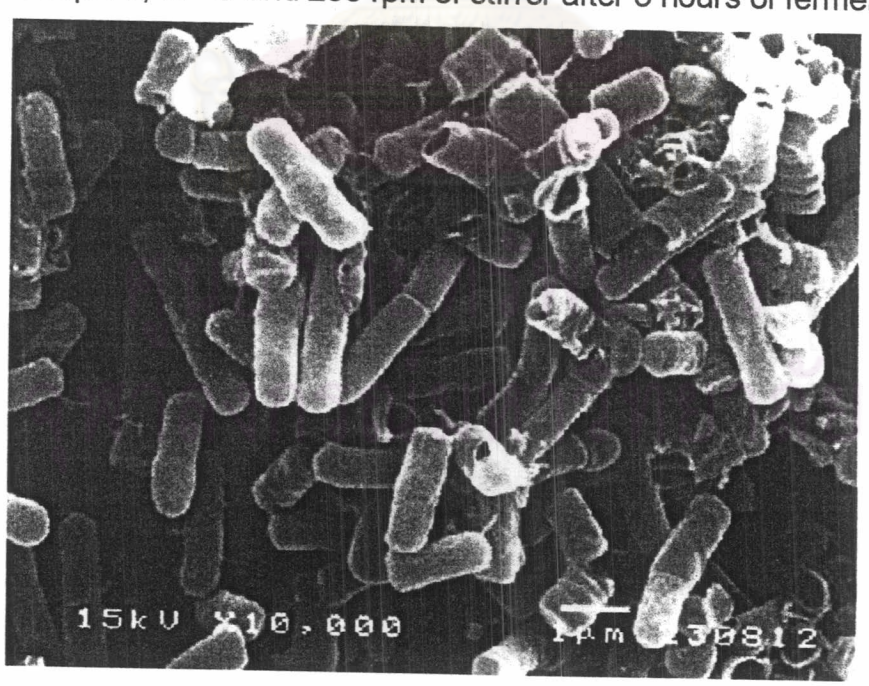

Figure 4.35: The shape of cell in ATPs obtained by point A2 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 5 hours of fermentation.

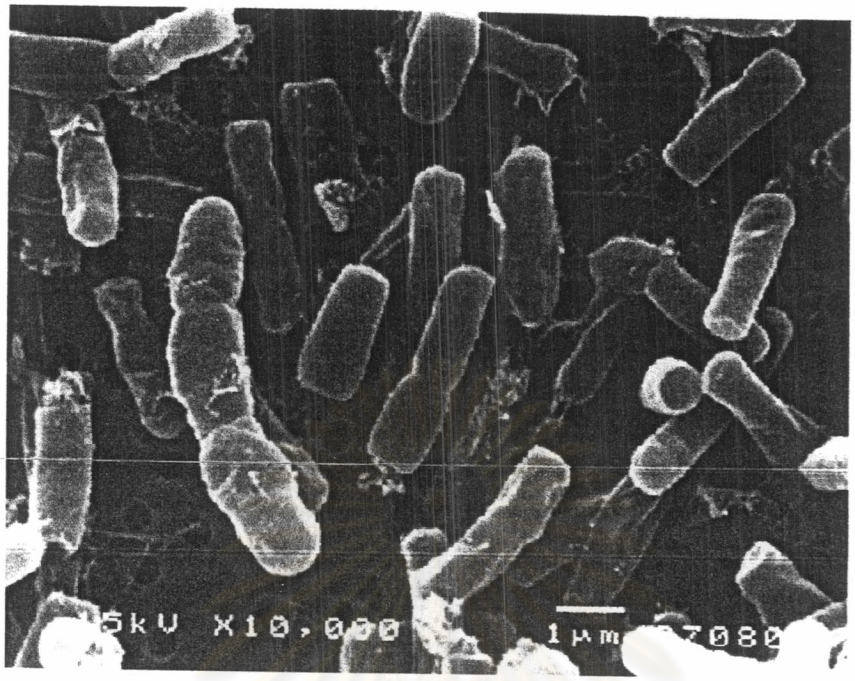

Figure 4.36: The shape of cell in ATPs obtained by point A3 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 5 hours of fermentation.

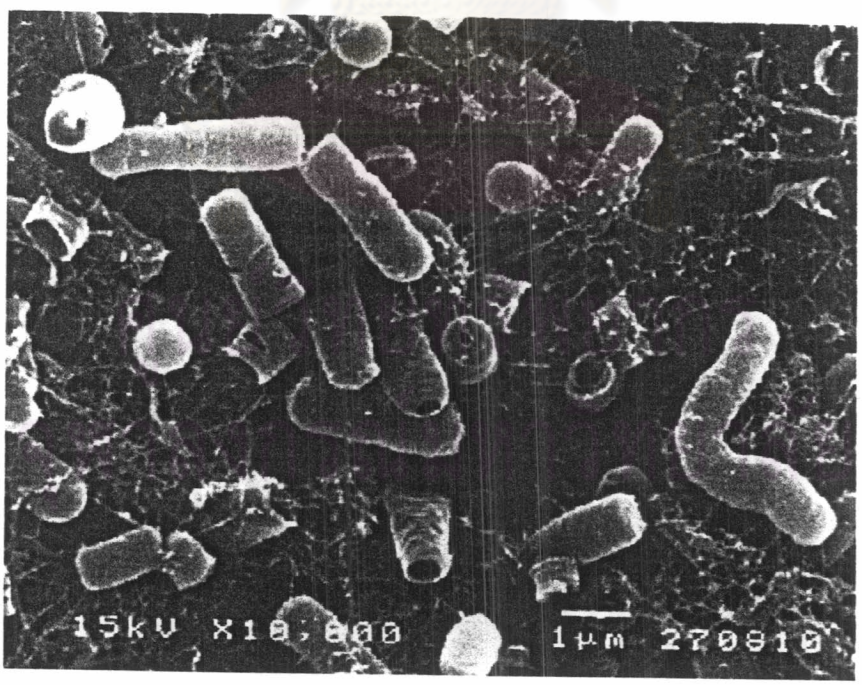

Figure 4.37: The shape of cell in ATPs obtained by point A4 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 5 hours of fermentation.

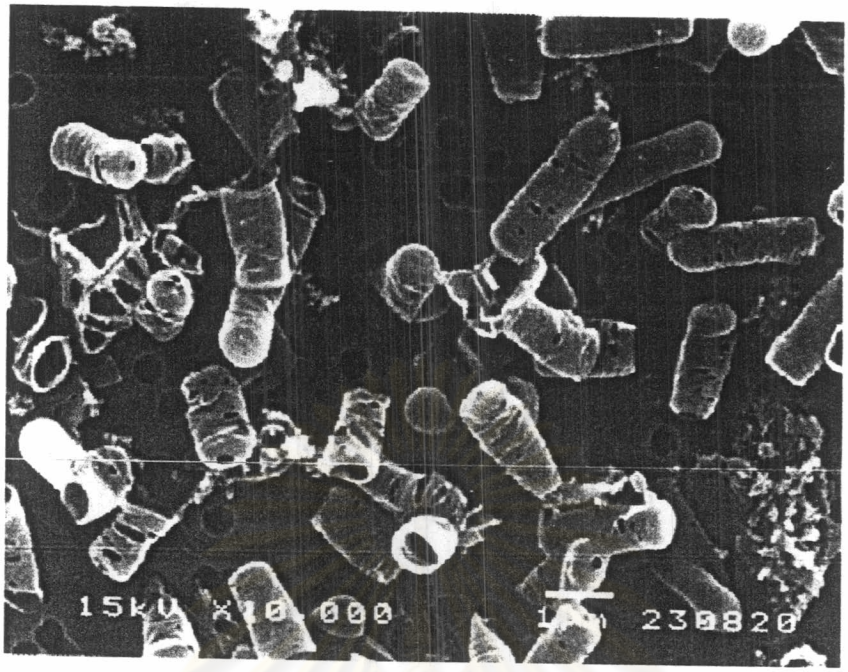

Figure 4.38: The shape of cell in conventional fermentation. Conditions: at pH 7, 37° C and 250 rpm of stirrer after 5 hours of fermentation.

After 5 hours of inoculation, the shape of cell in ATPs obtained by A1 and A2 was healthy and normal (figure 4.34, 4.35) when that in A3, A4 seems occur a change in the surface of cell. Figure 4.36 illustrated that interface of cell was quite rough with some cells elongated and others to be shorter than normal. That would also see in figure 4.37.

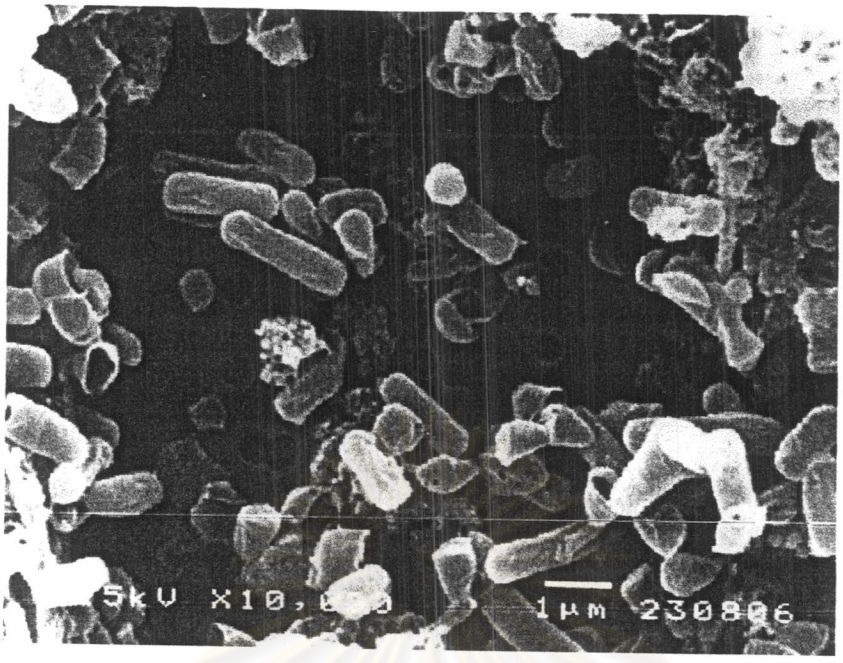

Figure 4.39: The shape of cell in ATPs obtained by point A1 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 24 hours of fermentation.

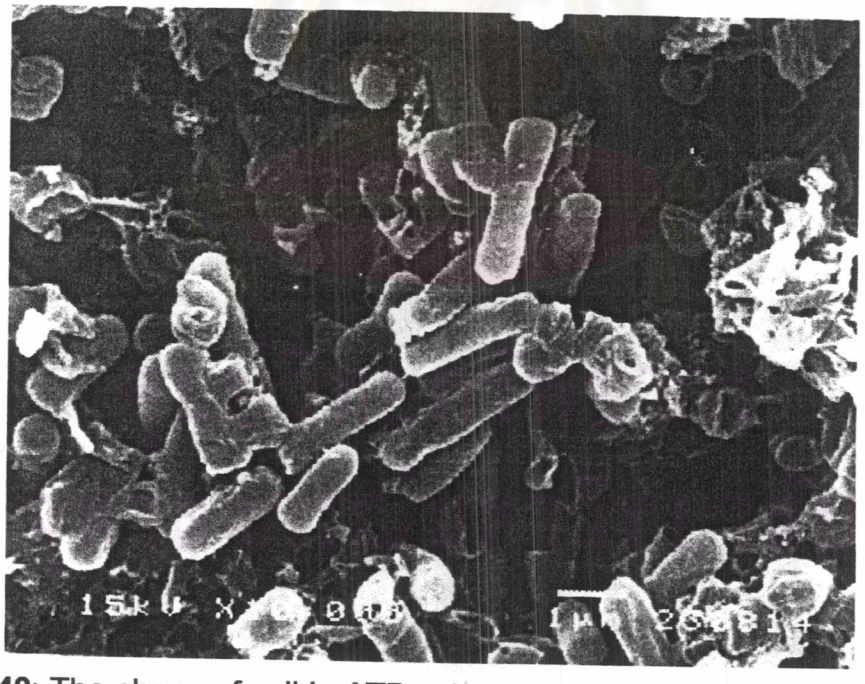

Figure 4.40: The shape of cell in ATPs obtained by point A2 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 24 hours of fermentation.

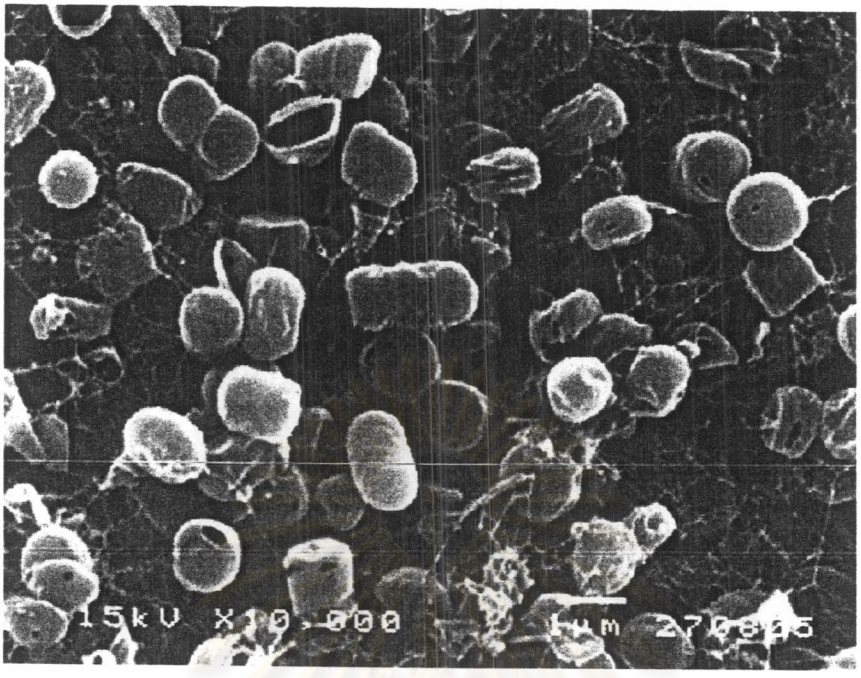

Figure 4.41: The shape of cell in ATPs obtained by point A3 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 24 hours of fermentation.

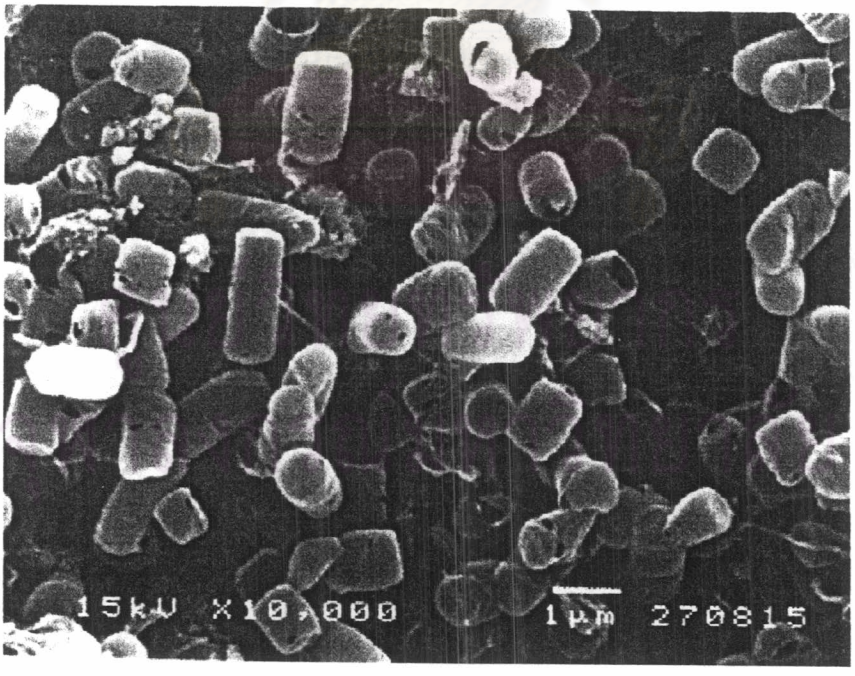

Figure 4.42: The shape of cell in ATPs obtained by point A4 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 24 hours of fermentation.

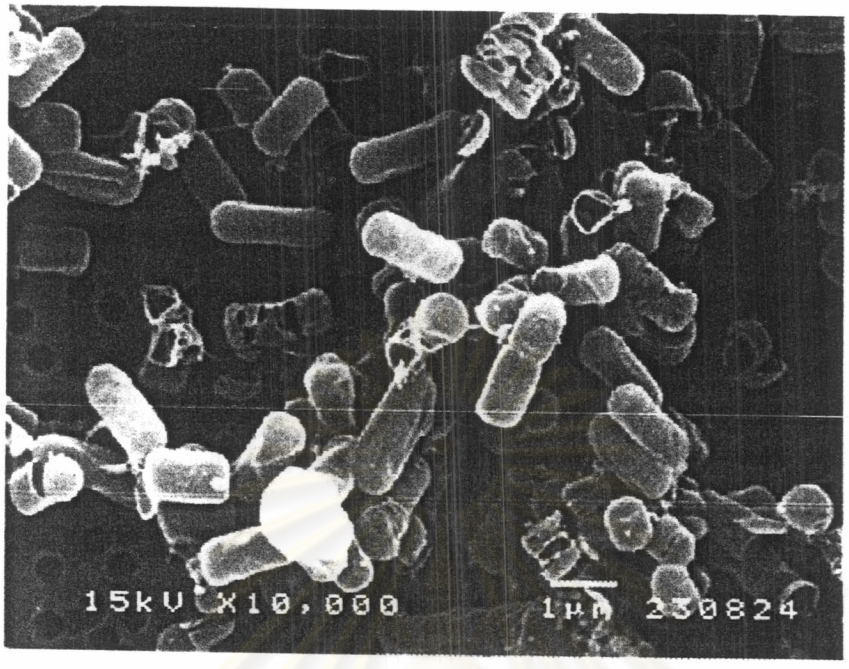

Figure 4.43: The shape of cell in conventional fermentation. Conditions: at pH 7, 37° C and 250 rpm of stirrer after 24 hours of fermentation.

Figures 4.38 and 4.39 shown a normal shape of cells as in conventional fermentation (figure 4.42) in ATPs which obtained by points A1 and A2 (table 4.5) respectively. The shape of cell was changed from rod to the sphere shape. It may be explained by effect of fluid dynamic in fermentor that caused by air bust, agitator, etc.

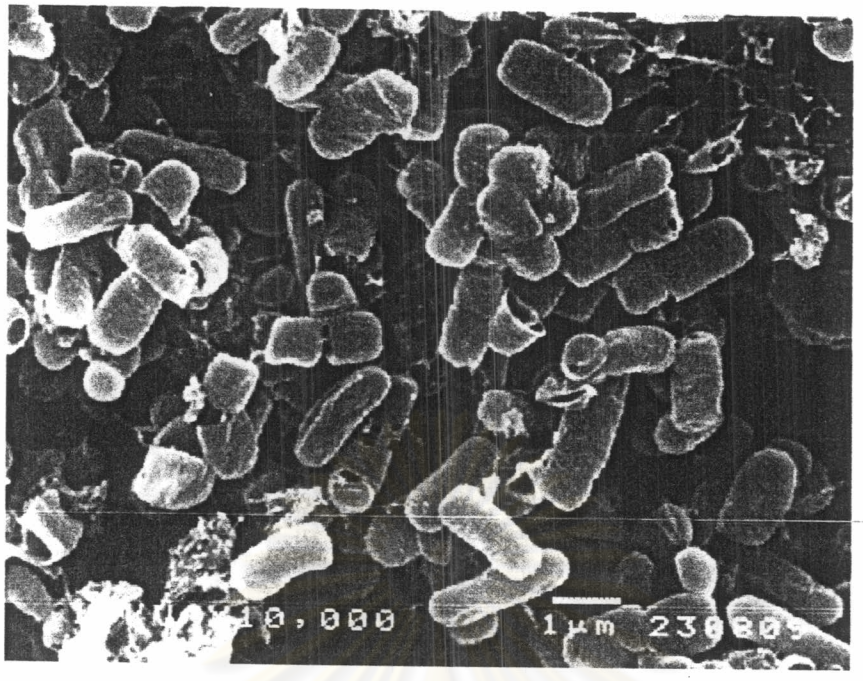

Figure 4.44: The shape of cell in ATPs obtained by point A1 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 48 hours of fermentation.

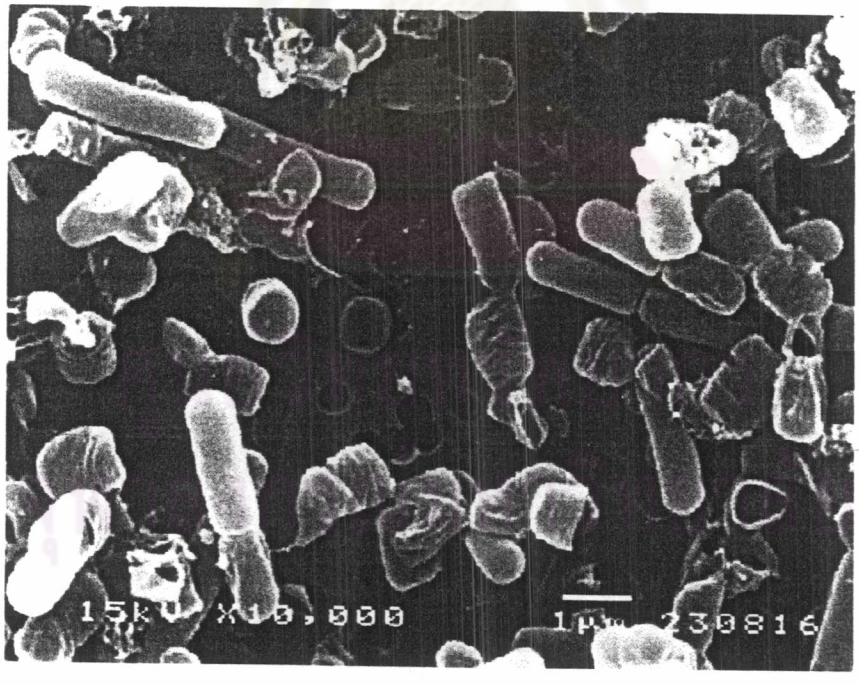

Figure 4.45: The shape of cell in ATPs obtained by point A2 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 48 hours of fermentation.

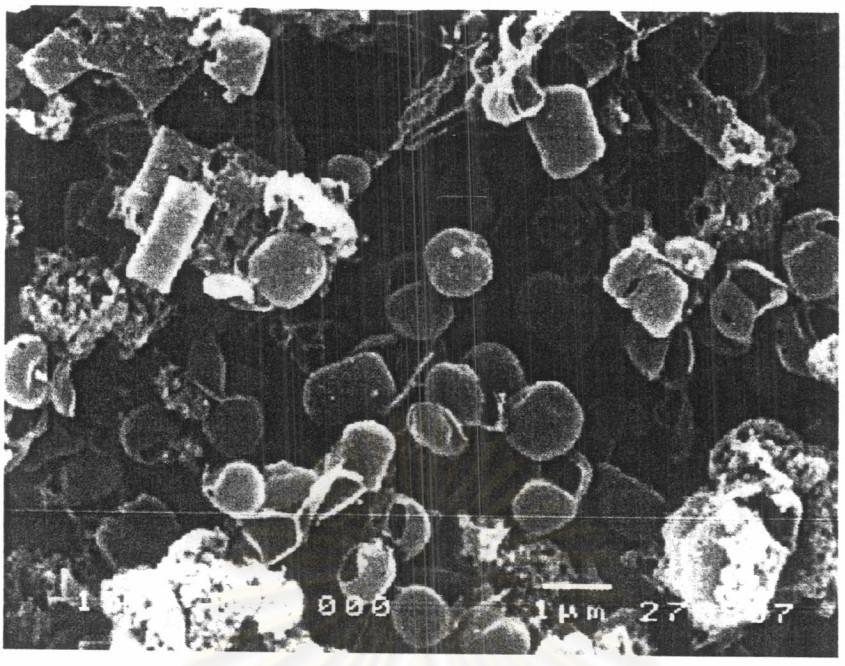

Figure 4.46: The shape of cell in ATPs obtained by point A3 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 48 hours of fermentation.

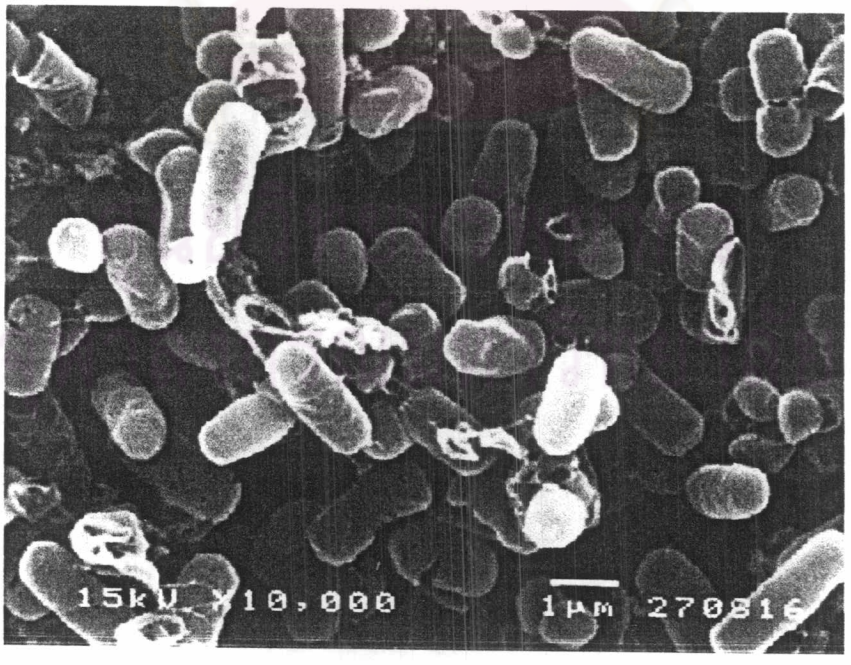

Figure 4.47: The shape of cell in ATPs obtained by point A4 given in Table 4.5 Conditions: at pH 7, 37° C and 250 rpm of stirrer after 48 hours of fermentation.

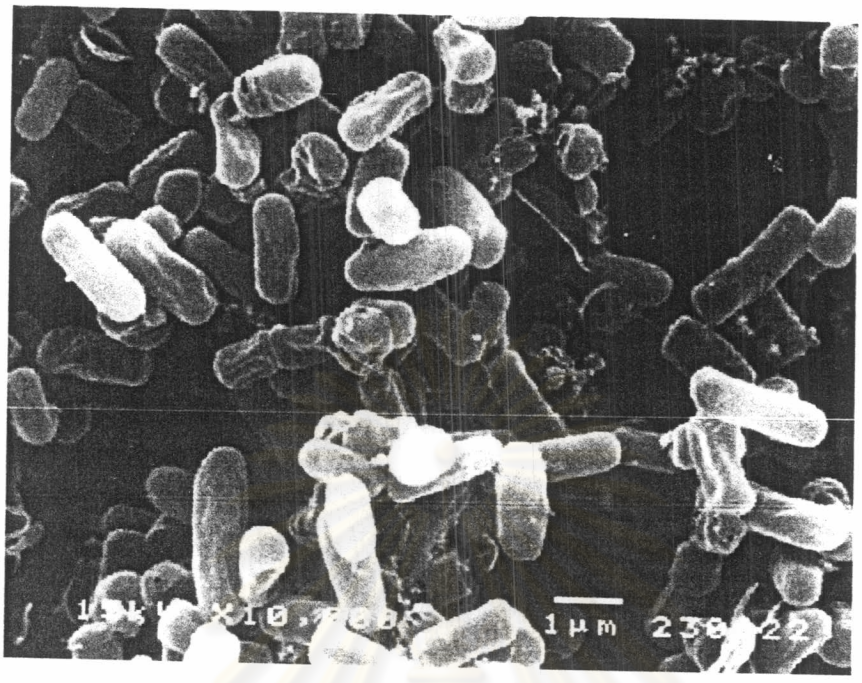

Figure 4.48: The shape of cell in conventional fermentation Conditions: at pH 7, 37° C and 250 rpm of stirrer after 48 hours of fermentation.

After 48 hours of inoculation, in the ATPs obtained by point A1, A2 given in table 4.5, the shape of cell still look healthy with some shorter rod occurred that the same in normal conventional fermentation (figure 4.43,4.44 and 4.47) when its in the A3 and A4 contained much more sphere shape (figure 4.45 and 4.46).

Generally, in all ATPs, the shell of dead cells always associated with polymer to make a gel net in the bottom phase that caused a difficulty in cell separation for the bottom phase and also protein, alkaline protease activity analysis. And we can conclude that with the lower total amount of PEG 4000 in the aqueous two-phase system the shorter rod cell was occurred more.