

The Potential of Strain M Protease in Degradations of Protein in Natural Rubber Latex

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Abstract—Strain M was isolated from the latex of *Hevea brasiliensis* that grow in the rubber farm area of Malaysia Rubber Board. Strain M was tentatively identified as *Bacillus* sp. Strain M demonstrated high protease production at pH 9, and this was suitable to be applied in rubber processing that was in alkaline conditions. The right and suitable proportion to be used in applying supernatant into the latex was two parts of latex and one part of enzyme. In this proportion, the latex was stable throughout the 72 hours of treatment. The potential of strain M to degrade protein in the natural rubber latex was proven with the reduction of 79.3% nitrogen in 24 hours treatment. Centrifugation process of the latex before undergoing the treatment had increased the protein degradation in latex. Although the centrifugation process did not achieve zero nitrogen content, it had improved the performance of protein denaturing in the natural rubber.

Keywords—*Hevea brasiliensis*, *Bacillus* sp., protease, latex.

I. INTRODUCTION

PROTEASES are an important class of microbial enzymes, which constitute about 65% of the total worldwide enzyme sales [10]. Proteases are widely used in several industrial factors such as in the detergent industry, food industry, in pharmaceutical and medical diagnosis as well as in textile industry and leather processing [11]. Proteases also have a potential to contribute in the development of high value added products due to their characteristic nature of aided digestion [12]. In this research study, protease is being used to degrade protein in the latex of *Hevea brasiliensis* to obtain modified natural rubber. Basically in a milky fluid of *Hevea brasiliensis* contains 30%-40% of the rubber hydrocarbon particles suspended in a serum together with a few percent of other non-rubber substances such as proteins, lipids, carbohydrates, sugars, some metal (non-rubber fraction), and the remaining major component is water [18]. Protein is present in fresh *Hevea brasiliensis* latex to the extent of approximately 1-1.5% by weight, of which about half is associated with the rubber. After the latex has been coagulated

with acid, a commercial sample of dried rubber typically contains about 1.5% protein. This protein and other non rubber components absorb water and give rise to a variety of undesirable effect such as reduced modulus and increased creep in the end product.

On our previous research, we had successfully identified fifteen isolates of bacteria protease producer. Among the isolates, three strains showed good potential as protease producer were name as strain M and strain O and strain K. Strain K showed high protease production at neutral condition and strain M worked best at pH 9. Both of these strains have their own plan of action in producing deproteinised natural rubber. However, for the purpose of degradations protein latex in rubber processing factory, we gave more focus to strain M. Strain M demonstrated high protease production at pH 9, and this was suitable to be applied in rubber processing that was in alkaline conditions.

Therefore, we had continued our research by applying the protease of strain M in natural rubber latex to see its performance to degrade protein. As a comparison, we used *Bacillus subtilis* ATCC 6633 to compare their performance to degrade protein in the latex.

2. Methodology

2.1 Strains and culture

Strain K, Strain M and strain O was isolated from the latex of *Hevea brasiliensis* that grow in the rubber farm area of Malaysia Rubber Board. The strain was tentatively identified as *Bacillus* sp. Strain M was grown in 250ml flasks containing 50 ml of 0.5% glucose, 0.75% peptone, 0.5% MgSO₄, 0.5% KH₂PO₄, and 0.01% FeSO₄ at 37°C in 250 rpm. *Bacillus subtilis* ATCC 6633 also were grown in same medium.

2.2 Protease assay

5ml of Casein (0.65%w/v, that is prepared in potassium Phosphate buffer pH 8) was pipette into a test tube. The casein solution was equilibrating to 37°C before added 1ml of enzyme (supernatant). Then, the casein and enzyme solution was mix by swirling and incubate at 37°C for 20 to 30 minutes. After that, 5ml of 110mM TCA was added in the solution. For blank, 5ml of TCA and 1ml of enzyme (supernatant) was added into the test tube that contains 5 ml of incubated casein. After that, the blank was mixed by swirling and incubate at 37°C for about 30 minutes. After 30 minutes

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of reaction, the solution was centrifuged and 2ml of the supernatant was used for color development.

In color development test, 2ml of the supernatant was added with 5ml 500mM Sodium Carbonate solution and 1ml of Folin Ciocalteu's Phenol Reagent. The solution was again mix by swirling and incubates at 37°C for 30 minutes. The absorbance was read at 660nm. The Enzyme activity was measured using tyrosine (0-796.4µg) as standard.

2.3 Protease activity at different pH value

The influence of pH on the proteolytic activity was investigated by adjusting the selected media to different initial pH using 6N NaOH for pH 7, pH8, pH 9, and pH 10. The sample cultures were withdrawn aseptically from the fermentation broth at appropriated time periods and were check for the OD, and protease concentration. The samples were properly keep in the sterile appendorf tubes and were stored in the freezer.

2.4 Protease activity at different temperature value

The influence of temperature on the proteolytic activity was determined by inoculating the inoculums of selected culture into 50 ml of selected medium and incubating at 30°C, 37°C, 45°C, and 55°C at 200 rpm for 24 hours. The sample cultures were withdrawn aseptically from the fermentation broth at appropriated time periods and were check for the OD, and protease concentration. The samples were properly keep in the sterile appendorf tubes and were stored in the freezer.

2.5 Protease activity at different agitation speed

The influence of the agitation speed on the protease enzyme synthesis was determined by inoculating the inoculums of selected culture into 50 ml of selected medium and incubating at 100rpm, 200 rpm and 300 rpm under the same fermentation conditions. The sample cultures were withdrawn aseptically from the fermentation broth at appropriated time periods and were check for the OD, and protease concentration. The samples were properly keep in the sterile appendorf tubes and were stored in the freezer.

2.6 Enzymatic deproteinisation of latex

Samples of fresh field latex with dry rubber content 33.4% were adjusted to pH 9 with dilute ammonia solution (0.3%) and stabilized by the addition of 0.2% phr Hydroxylamine neutral sulphate (HNS) and 1.35% phr Polyoxyethylene 10 phenol ether (N10). Stabilized latex was incubated with enzyme (supernatant) at 37°C and 64 rpm. In this experiment we divided the supernatant (enzyme) into three proportions. Proportion A contains one part of latex and one part of enzyme. Proportion B contains two part of latex and one part of enzyme and proportion C contains two part of latex and three part of enzyme. For the control, same proportion had been set up, instead of using supernatant (enzyme), medium A that contained of 0.5% glucose, 0.75% peptone, 0.5% MgSO₄, 0.5% KH₂PO₄ and 0.01% FeSO₄ had been used to performed condition without protein degradation of the latex. The

enzymatic hydrolysis was allowed to carry on for 72 hours. 20ml samples were withdrawn at pre-determined times for further analysis.

2.7 Rubber processes

5% strength of formic acid solution was added to the 30ml latex in the beaker until the ph of the latex decrease to pH 6.5 and then swirled well, then left for 5 minutes. The samples then heated over the steam bath for 10 to 15 minutes until a clear serum was obtained. This was an indication of a complete coagulation. After that, the sample was removed from the steam bath, a small amount of distill water was added to prevent it from drying up, and allowed to cool down. Then, the coagulum was removed and crept using creeper. The dry rubber was washed with distilled water and this creeping and washing process were repeated for 5 to 6 times. The dry rubber was soaked in the distill water and washed to make sure the entire nitrogen was removed. After washing procedure was finish, the dry rubber was dried in the hot oven at 70°C for 16 hours (preferably overnight until a clear rubber film was obtained).

2.8 Nitrogen test

0.1g of the rubber sample was weighted accurately into a Micro Kjeldahl Flask and 0.65g of catalyst mixture and 2.5ml of concentrated sulfuric acid (H₂SO₄) were added into the flask slowly. Then the flasks placed on the Kjeldahl Nitrogen Digestion Apparatus and were boiled gently until the digest became clear green in color or colorless with no yellow tint. Normally it required 1 hour. Then after boiling, the flask was removed from the digestion unit and kept at room temperature until the digest was cooled and diluted with 10ml water. Transfer with two or three 3ml portions of water to distillation apparatus which has been previously steamed out for 30 minutes. 10ml of boric acid solution (2%) and two or three drops of screened methyl red indicator was added to the 100 ml receiving conical flask. 10 ml of 67% NaOH solution was added to the distillation vessel and washed it down with not more than 5 ml of water. Steam was passed through the distillation apparatus for 5 minutes from the time the distillate begins to come over. Lower the receiver until the condenser tip was well above the solution and continues distilling for a further minute. The end of the condenser was wash with water. After completion of distillation, the distillate was titrated with standardized 0.01N sulphuric acid immediately using a 10 ml microburette. The color will change from green to a light violet.

2.9 Centrifugation process of latex

Samples of fresh field latex were adjusted to pH 9 with dilute ammonia solution (0.3%) and stabilized by the addition of 0.2% phr Hydroxylamine neutral sulphate (HNS) and 1.35% phr N10. The latex was put in the 50ml centrifuge tubes and centrifuged at 10000 rpm for 20 minutes. The latex was collected for further analysis.

3. Result and discussion

3.1 Effect of physical properties on protease production of strain K, strain M and strain O

3.1.1 Effect of pH on enzyme production

In rubber industry, latex must be maintained at high pH to stabilize it throughout the rubber processing process. The high pH will control the bacterial and enzymic metabolic activities in latex. By controlling these, the metabolism of non-rubber substrates into acids that will turn the latex into the coagulated form can be prevented [4]. Therefore, the latex will remain in a liquid phase. Proteases that will be used in rubber processing should have a maximum activity in the alkaline region [1]. Figure 3.1.1 shows that, all of these strains showed protease production in alkaline medium (pH8 to pH10). Strain K and Strain O, demonstrated similar pattern, which showed good protease production at pH 7. The biomass production and enzyme production gradually decreased from natural to alkaline medium. At pH 10, the enzyme production still can be detected although in low value. Strain K demonstrated 62%, 87% and 96% reduction of protease production and strain O demonstrated 33%, 63% and 83% reduction of protease production at pH 8, pH9 and pH10 respectively. Therefore these two strains, strain K and strain O were not recommended to be applied in latex processing as the latex should be maintained at alkaline condition. However these two strains have valuable potential as a protein degrader in neutral condition and can be applied directly to the fresh field latex but need further attention to acquiring knowledge for the right approach. From figure 3.1, strain M worked best at pH 9 that showed 21% and 57% increment in protease production at pH 8 and pH 9 respectively. However, it showed 49% reduction of protease production at pH 10 compared to pH 7. Comparing the potential of strain M as alkaline protease bacteria from previous research findings, strain M worked in a narrower level of alkaline pH that was in pH 8 and pH9. As most of previous research findings, their strains maintained high activity at pH higher than pH9 [6]. However, our findings showed that pH9 as the optimum pH for protease production for strain M and it was considered good and suitable as the pH in the real latex processing was 9.

Therefore, from the pH profiling of strain M, it had successfully suggested us, to pick strain M as the best strain to be applied in latex processing compared to strain K and strain O.

3.1.2 Effect of temperature on protease production

It was reported that there was a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake [28]. In this experiment, the effect of the incubation temperature on protease production by strain K, strain M and strain O were investigated at temperatures ranging from 30°C to 55°C.

Strain K and strain O in Fig. 3.2 demonstrated similar trend in the temperature profiling. When the temperature increased from 30°C to 37°C, the enzyme also increased. The protease production decreased drastically with 81% and 92% at 45°C for strain K and strain O respectively. At 55°C, strain K and

strain O could not grow at all. Strain M seems much more tolerable with the higher temperature compared to strain K and strain O. The biomass production increased from 30°C to 37°C. However, enzyme productions were dropped at 45°C. At 45°C, there is 7.3% reduction of protease production compared at 37°C. At 55°C, the presence of protease still can be detected although it was very little.

The fall of protease production at higher temperature was related to the enzyme properties. As for the extra-cellular enzymes, temperature was found to influence their secretion, possibly by changing the physical properties of the cell membrane [19]. On the other hand, a lower growth of those strains at high temperatures could be due to the lack of dissolved oxygen in the medium, which resulted to a low protease activity. It is a well-known fact that protein conformation changes or degraded at higher temperatures, and hence, causes a decrease in the protease activity [10].

These results obviously showed that all of these strains had optimum enzyme production at 37°C. Several investigators reported maximum protease production from *Bacillus* spp. at 35°C-37°C. Therefore, we suggested that all of these strains were identified as mesophilic bacteria. Higher temperatures were not suitable for the growth of those strains especially strain K and strain O. Strain M however can survive at 45°C but not at higher temperature.

In rubber processing industry, the enzyme application exposed at 60°C to reach its optimum activity. The high temperature tend to increase the energy consumption thus may cause high cost in producing DPNR. Considering the potential of our strains that had optimum protease activity at 37°C, there was a good sign of cutting the production cost of DPNR in term of energy consumption

3.1.3 Effect of agitation on protease production

Micro-organisms vary in their oxygen requirements. In particular, oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. The agitation speed also influences the extent of mixing in the shake flasks or in the bioreactor and thus affects the nutrient availability to microorganism. [2], [7], [15]. It was reported that, protease production was drastically inhibited at low agitation rates [2]. The enhancing effect of agitation on protease production had been reported in *Bacillus licheniformis* S40 which required agitation at 200 rpm for maximum production [22]. The agitation speed reported in the case of *Bacillus* sp. [23] were 300 rpm.

Fig. 3.3, it showed the protease production from all of the three strains were increased steadily with the increased of agitation speed up to 200 rpm. However, all of these three strains showed fall in enzyme production when the agitation speed was increased to 300 rpm. The protease production decreased about 31%, 49% and 67% for strain K, strain M and strain O at the agitation speed of 300 rpm. Despite the markedly fall of enzyme production, the biomass productions were not much effected at 300 rpm. Theoretically, the enzyme production should also be stable as the biomass production

was not much affected. The best agitation speed that supported maximal enzyme production by these strains was at 200 rpm.

Therefore, the fall of enzyme production at the higher agitation speed was subjected to the enzyme degradation or the lyses of the enzyme molecule, thus made it lost their activities. It has been reported that enzymes are also susceptible to mechanical force, which may disturb the elaborate shape of complex molecules to such a degree that denaturation occurs [3, 6, 9]. High agitation speed caused a drop in enzyme production due to cell lysis or excessive cell permeability related to abrasion by shear forces and by oxygen limitation in dense pellets formed the formation of extracellular polysaccharides [27].

As a conclusion, higher agitation rates could increase the oxygen pressure of the system but did not bring about the increase in production, probably because at a high agitation speed, the structure of enzyme would be altered [20]. However, lowering the aeration rate could cause a drastic reduction in the protease yields [24]. This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis [17].

3.2 Protease production by strain M

We had pick strain M as the best strain to be applied in latex processing compared to strain K and strain O based on their good performance in producing protease in alkaline condition especially in pH 9. Latex must be maintained in alkaline condition to ensure its stability. We had identified the suitable medium for strain M to produce higher protease compared in nutrient broth medium. Medium A that contain 0.5% glucose, 0.75% peptone, 0.5% KH_2PO_4 , 0.5% MgSO_4 and 0.01% FeSO_4 was found to be the suitable medium for protease production with improvement almost 219% compared to the cultivation in control medium (data not showed). Crude protease of strain M in medium A was used to be applied in natural rubber processing.

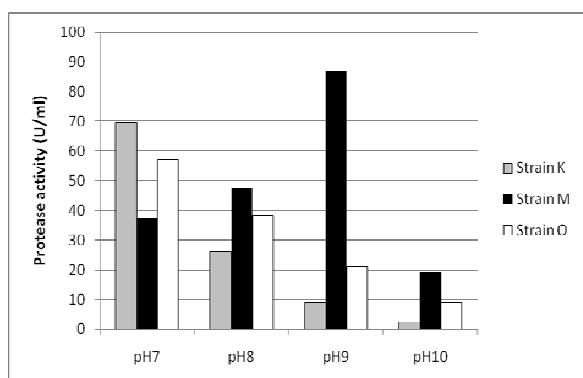


Fig. 3.1 The protease production of strain K, strain M and strain O in different pH medium. Experiments were carried out using the nutrient broth medium at 37°C. Results represent the means of two experiments. Protease activity at pH 7 was considered as 100%.

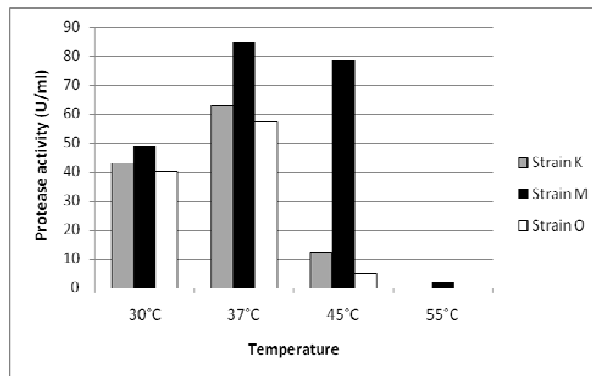


Fig. 3.2 The protease production and of Strain K, strain M and strain O at different temperature. Experiments were carried out using the nutrient broth medium with initial pH 7 for strain K and O and initial pH 9 for strain M. Results represent the means of two experiments. Protease activity at 37°C was considered as 100%.

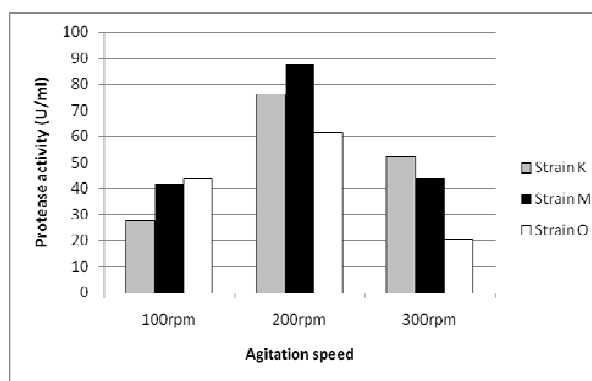


Fig. 3.3 The protease production of Strain K, strain M and strain O at different rpm. Experiments were carried out using the nutrient broth medium at 37°C with initial pH 7 for strain K and O and initial pH 9 for strain M. Results represent the means of two experiments. Protease activity at 200 rpm was considered as 100%.

3.3 Determination of right crude enzyme proportion in latex for protein denaturing process

We decided to use crude protease in the supernatant form to be applied into the latex. From earlier reported research they had diluted the latex first before adding protease. The latex had diluted with deionised water to 10% of dry rubber content before added Alcalase, a commercial enzyme as alkaline protease [16]. Other had diluted the latex to 5% of dry rubber content before added the Superase, a commercial preparation enzyme containing Subtilisin [1]. The dilution less than 5% was suggested in order to ensure the products of digestion of the proteins mainly peptides were not occluded in the coagulum [25]. In our experiment, the proportion directly had diluted the latex to 16.7% DRC, 25% DRC and 8.35% DRC in proportion which were marked as A, B and C respectively.

In proportion A, protease from both strains showed latex auto coagulation within 24 hours of incubation and in proportion C both strains showed latex auto coagulation after

24 hours of incubation. In proportion B that was two part of latex and one part of enzyme showed a stable condition where the latex remained in colloidal condition within the 72 hours of incubation. The controls remained in colloidal condition within the 72 hours for proportion B but not in proportion A and C. The auto-coagulation phenomenon that occurred in this experiment was considered bad for latex processing as the latex was not totally reacted with the enzyme thus the protein in the latex has not completely hydrolyze.

There are a few theories reported on auto-coagulation of latex. First is the normal acid coagulation rubber that occurs largely due to the heavy contamination of bacteria. From the bacterial metabolic activities, subsequent breakdown of non-rubber will develop volatile fatty acids (VFA) in latex. VFA consisted mainly of acetic, formic and propionic acids. VFA will cause the pH value of the latex to fall and coagulated the latex [4]. Second theory is by thermal oxidative degradation in the latex. This theory involve the function of copper, an inorganic constituent in latex that act as pro oxidant. Copper in fresh latex might complex with proteins and amino acids and in this form, it does not impart any deleterious effect on the ageing of natural rubber. However, when protein and amino acids components of the copper complex being degrade, it will release free copper which is known as a very active prooxidant of natural rubber [8].

In this experiment, the chemically clean but not sterile apparatus such as the latex cup and the latex itself were used. Therefore, there were chances of bacteria proliferation at the early stage of the incubation. However the ammonia solution was added to preserve the latex by keeping the bacterial population from increasing. Moreover, if the auto-coagulation was caused by the bacteria contamination, proportion B should also be affected as this proportion also came from the same source of latex. The control of both proportion A and C were also demonstrated auto-coagulation within 24 hours of incubation had proved that there were a reason of proportion that made the latex unstable, thicken and partially coagulated.

Therefore, the right and suitable proportion to be used in applying supernatant into the latex was two parts of latex and one part of enzyme. In this prolong treatment that take 72 hours of incubation time, the protein molecules on the surface of natural rubber latex and in the serum were hydrolyzed by the protease. The 20ml of samples were withdrawn at every 24 hours. These samples were added with 5% strength of formic acid solution until the pH of the latex decreased to pH 6.5. To coagulate the latex, the samples then heated over the steam bath for 10 to 15 minutes until a clear serum obtained. After cooling, the coagulum had been crept and washed up to six times before soak in the distilled water. In this process, hopefully the entire nitrogen should be removed. Then the samples we dried in the hot oven at 70°C for 16 hours (preferably overnight until a clear rubber film was obtained). The clear rubber film was cut in small portion and underwent the Kjeldahl technique for the determination of organic nitrogen.

After performing the nitrogen test using Kjeldahl technique, proportion B of strain M in Figure 3.4 showed 0.24% of nitrogen within 24 hours of treatment and the percentage of nitrogen reduced until 0.125% after 72 hours. It seem that within the 24 hours the nitrogen content had decreased to 88% and prolong treatment did not give tremendous effect in degrade the protein. The control remained 1.1% of nitrogen. In the proportion A and C, although there were coagulated after 24 hours, there still showed decreases in nitrogen content about 0.45% and 0.36% nitrogen content respectively.

Fig. 3.5 showed that *Bacillus subtilis*, demonstrated same result, which proportion B became the right proportion that maintain in colloidal stage throughout the 72 hours of treatment. In proportion B, the nitrogen content was 0.29% at 24 hours of treatment and remained in that value until 72 hours of treatment.

Comparing the potential as protease producer that can degrade the protein in the latex, we had decided that both of these strains had a good ability and gave a good effect to degrade protein in the latex. Our strain, which was strain M showed slightly better performance than *Bacillus subtilis* where the nitrogen content was decreased at each 24 hours of treatment, however using supernatant from *Bacillus subtilis*, the nitrogen decreased until 24 hours treatment and the prolong treatment seem not have much effect in the nitrogen content.

Therefore, the lowest level of nitrogen content of 0.125% was attained by this protease action of strain M was assumed good for protein denaturing in latex.

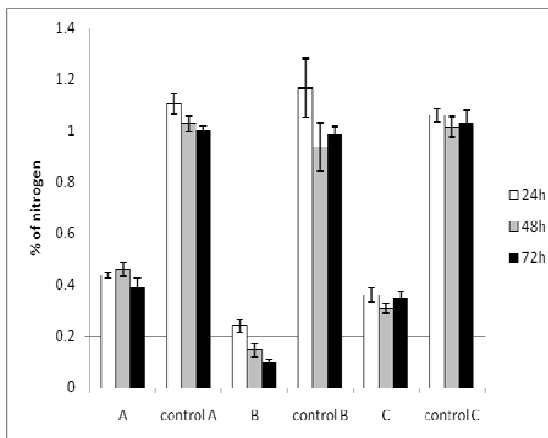


Fig. 3.4: The digestion of rubber protein with crude protease of strain M.

3.4 Latex improvement by centrifugation process for better protein denaturing

To improve the protein denaturing in latex, centrifugation process of the latex before it was treated with the enzyme was decided. By centrifugation, it will separate unwanted substances from the latex, thus make the latex more concentrate and the enzyme will work better.

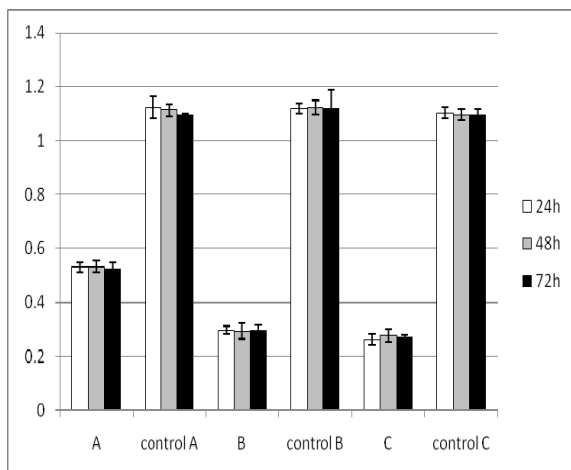


Fig. 3.5: The digestion of rubber protein with crude protease of *Bacillus subtilis*. For both experiment in graph 3.8.1a and 3.8.1b, the crude protease was divided into 3 proportions. Proportion A contains one part of latex and one part of enzyme. Proportion B contains two part of latex and one part of enzyme and proportion C contains two part of latex and three part of enzyme. Results represent the means of three experiments, and bars indicate \pm standard deviation.

Theoretically, fresh latex may be divided into three main fractions by centrifugation. These are a white upper layer, an aqueous serum and the so-called 'bottom fraction' [5, 14, and 9]. The top layer comprises rubber hydrocarbon particles stabilized by an adsorbed layer of protein and phospholipids. The serum contains most of the soluble substances normally found in plant cells, including amino acids, proteins, carbohydrate, organic acids, inorganic salt, and nucleotidic materials. The bottom fraction consists largely of the lutoid particles, varying amounts of rubber, lipid, mitochondria and other particulate components of normal plant cells having a density greater than of the serum examples ribosomes [9, 21, 13].

The total protein content of fresh latex is approximately 1%, which about one-fifth is adsorbed at the surface of the rubber particles and some two-thirds are dissolved in the aqueous serum. The remainder is associated with the larger particulate bodies in latex, known collectively as bottom fraction, with contain little rubber and sediment on centrifugation.

Wentworth in his research study, points out that enzyme prefer to attack protein in the serum, thus reducing decomposition of the protein in the rubber phase [25]. Therefore, if the concentration of the serum constituents can be reduce, better decomposition of protein in the rubber phase will be achieve. Smith also suggested that centrifugation will remove some enzyme inhibitor which was probably associated with the heavy fraction of the latex [26].

In this experiment, the natural rubber latex was centrifuged for 30 minutes at 10000 rpm. The supernatant were collected and leave the pellet. The dry rubber content of the centrifuged latex was 48.2%. The latex then stabilized with diluted

ammonia solution (0.3%) and stabilized by the addition of 0.2% phr Hydroxylamine neutral sulphate (HNS) and 1.35% phr N10.

In the previous experiment, proportion contained two part of latex and one part of enzyme became the stable condition which the latex remain in colloidal stage. Therefore in this experiment, the centrifuged latex was treated with crude enzyme in that proportion for 72 hours. Then 20ml of the samples were taken at every 24 hours for nitrogen analysis.

The slightly fall of the nitrogen content in the rubber that was underwent the centrifugation process shown in Figure 3.6. At 24 hours of treatment with crude protease of strain M, the nitrogen content in the rubber from the latex that not being centrifuged was 0.26%. The value dropped to 0.11% when the latex was centrifuged before treatment with enzyme. After 72 hours, the centrifuged latex showed 0.09% of nitrogen content. Although the centrifugation process not achieves zero nitrogen content, it had improved the performance of protein denaturing in the natural rubber. Same result was demonstrated in Figure 3.7 with crude protease of *Bacillus subtilis* being used in the latex, the nitrogen content drop until 0.19% when the latex was centrifuged before treatment with enzyme

A possible explanation of this fall in nitrogen content shown by the latex concentrate lies in the removal of significant quantities of the protein, both in the serum phase and on the surface of the smaller rubber particles as a result of the centrifugation.

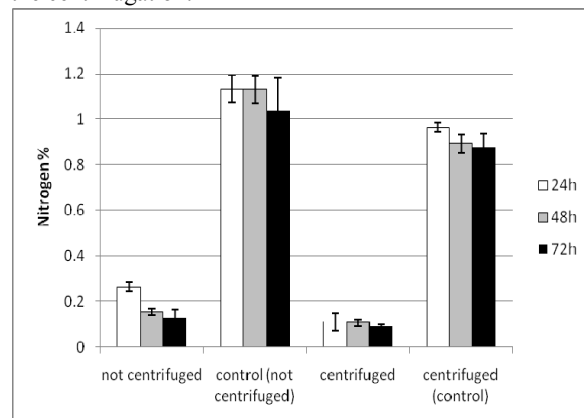


Fig. 3.6: The digestion of rubber protein with crude protease of strain M in proportion A that contains one part of centrifuged latex and one part of enzyme. Results represent the means of three experiments, and bars indicate \pm standard deviation.

VI. CONCLUSION

The optimization of physical properties had revealed the potential of strain M in producing optimum protease at pH 9, 37°C, and 200 rpm. The ability in producing higher protease in alkaline condition was important in latex application as natural rubber latex must be in alkaline condition to maintain its stability. Strain K and strain O, otherwise showed optimum protease at pH 7, 37°C, and 200 rpm.

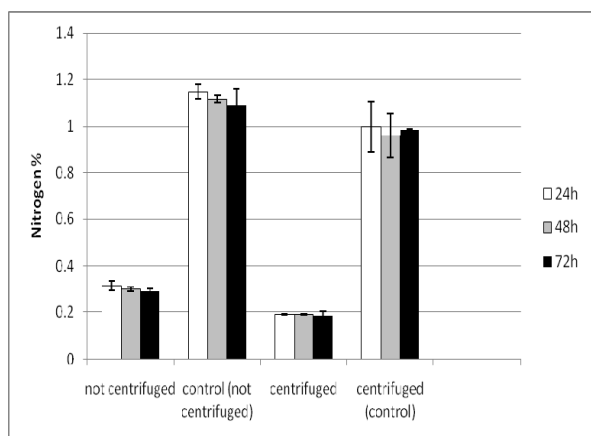


Fig. 3.7: The digestion of rubber protein with crude protease of *Bacillus subtilis* in proportion A that contains one part of centrifuged latex and one part of enzyme. Results represent the means of three experiments, and bars indicate \pm standard deviation.

The potential of strain M to degrade protein in the natural rubber latex was proven with the reduction of 79.3% at 24 hours treatment. The crude protease was applied with proportion of two parts of latex and one part of enzyme. In this proportion, the latex was stable in the colloidal condition. Although from the stability experiment the protease activity decrease at each 24 hours, the protease still acted well on degrading the protein in the latex. Prolong treatment for 48 hours and 72 hours had decrease the nitrogen content almost 95%. Centrifugation process of the latex before undergo the treatment had increased the protein degradation in latex. This result was successfully produce deproteinised natural rubber. However, more research should be done to investigate the quality of the rubber obtained from the treatment of strain M protease. Good quality rubber should have excellent film-forming property and green strength of its raw rubber film.

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REFERENCES

- [1] B.L. Archer, "Biochemistry of enzymic deproteinisation of *Hevea brasiliensis* latex", *Proceedings of the International Rubber Conferences*, Kuala Lumpur, Malaysia, 1975, pp:54.
- [2] Q. K. Beg, V.Sahai, and R. Gupta, "Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in a bioreactor", *Process Biochemistry*, 39:pp203-209, 2003.
- [3] U.C. Banerjee, R.K. Sani, W. Azmi, R. Soni, "Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive", *Process Biochem*, 35: pp213-219, 1999.
- [4] K. J. Cherrappathanathu, "Microbial studies of *Hevea latex* with Particular reference to its keeping quality and coagulation", Thesis for the Degree D. Sc. (Agric.) to the University of Gent, Belgium, 1977.
- [5] A. S. Cook, and B.C. Sekhar, "Fraction from *Hevea brasiliensis* latex centrifuged at 59 000g", *J. Rubb. Res. Ins. Malaya*, 14, pp163, 1953.
- [6] R. Gupta, Q.K. Beg, and P. Lorenz, "Bacterial alkaline protease: molecular approaches and industrial application", *Appl. Microbial. Biot.* 59: pp15-32,2002.
- [7] A. Hameed, T. Keshavarz, C.S. Evan, "Effect of dissolved oxygen tension on pH on the production of extracellular protease from a new isolated of *Bacillus subtilis* K2, for use in leather processing". *J Chem Technol Biotechnol*, 74:pp5-8, 1999.
- [8] H. Hasma, and Alias, "Role of some non rubber constituents on thermal oxidative Ageing of natural rubber", *J Nat. Rubb. Res.*, 5(1), pp1-8. 1990.
- [9] L. N. S. Homans, and G. E. Van Gils, "Fresh Hevea latex. A complex colloidal system", *Proc 2nd Rubber Technol. Conf.* London, Cambridge: W. Heffer and Sons Ltd., 1948, pp 292.
- [10] B. Johnvesly, and G.R. Naik, "Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus sp.* JB-99 in a chemically defined medium", *Process biochemistry*, 37: pp 139-144, 2001.
- [11] H.S. Joo, G.C. Kumar, G.C. Park, S.R. Paik, C.S. Chang, "Oxidant and SDS stable alkaline protease from *Bacillus clausii* I-52: production and some properties", *J Appl Microbial.*, 95:pp 267-72, 2003.
- [12] C.G. Kumar, H. Takagi, "Microbial alkaline protease. From a bioindustrial viewpoints". *Biotechnol. Adv.*, 17, pp561-594, 1999.
- [13] A. I. McMullen, *Journal of the Rubber Research Institute of Malaya*, 13:29, 1951.
- [14] G. F. J. Moir, "Ultracentrifugation and staining of Hevea latex", *Nature, Lond.* 184, pp1626, 1959.
- [15] S. H. Moon, and S. J. Parulekar, "A parametric study of protease production in batch and fed batch culture of *Bacillus firmus*", *Biotechnol Bioeng.*, 37: pp 467-483, 1991.
- [16] N. Ichikawa, A. H. Eng, and T. Yasutuki, "Properties of Deproteinised Natural Rubber latex". *Proceedings International Rubber Technology Conferences 1993 Kuala Lumpur, Malaysia.*, 1993.
- [17] W.C.A. Nascimento, M.L.L. Martins, "Production and properties of an extracellular protease from thermophilic *Bacillus sp.*", *Braz J Microbiol.*;35:91-96. doi: 10.1590/S1517-83822004000100015, 2004.
- [18] S.H. Ong, R. Rahman, "Breeding and selection of clonal genotype for climatic stress conditions". *Proceeding IRRDB Symp. Vol 1 General soil, Fertilization and Breeding & Selection.* Ho Chi Minh City, 1997, pp 149-154.
- [19] R.N.Z.A. Rahman, L.P. Geok, M. Basri, A.B. Salleh, "Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K", *Bioresource Technol.* 96: pp429-436, 2005.
- [20] S. Roychoudhury, S.J. Parulekar, W.A. Weigand, "Cell Growth and a-Amylase Production Characteristics of *Bacillus amyloliquefaciens*". *Biotechnol Bioeng.* 1988;33:197-206. doi: 10.1002/bit.260330209, 1998.
- [21] W. A. Southorn, (1961). "Microscopy of Hevea latex". *Proc. Nat. Rubb. Res. Conf. Kuala Lumpur*, 1960, pp766.
- [22] S. Sen, and T. Satyanarayana, "Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40". *Ind J Microbiol.* 33: pp43-47, 1993.
- [23] H. Takami, T. Akiba, and K. Horikoshi, "Production of extremely thermostable alkaline protease from *Bacillus sp.* AH-101". *Appl Microbial Biotechnol*, 30: pp120-124, 1989.
- [24] S.L. Wang, T. Y. Kao, C. L. Wang, Y. H. Yen, M. K. Chern, Y.H. Chen, "A solvent stable metalloprotease produced by *Bacillus sp.* TKU004 and its application in the deproteinization of squid pen for β -chitin preparation", *Enzyme Microb Tech*; 39:724-731. doi: 10.1016/j.enzmictec.2005.12.007, 2006.
- [25] V. H. Wenworth, "Improvement in the preparation of purified latex and the preparation of rubber therefrom". Br. Pat. No. 551 666., 1941.
- [26] J. F. Smith, *Treatment of rubber.* Br. Pat. Applic. No. 37 575/71, 1971.
- [27] I. Darah, and C.O. Ibrahim, "Effect of agitation on production of lignin-degrading enzymes by *Phanerochaete chrysosporium* grown in shake-flask cultures". *As. Pac. J. Mol. Biol. Biotechnol.*, 4, pp174-182, 1996.
- [28] J. Frankena, G. M. Koningstein, H. W. Van Verseveld, A. H. Stouthamer, "Effect of different limitations in chemostat cultures on growth and production of exocellular protease by *Bacillus licheniformis*". *Appl Microbiol Biotechnol.* 24:pp106-112, 1986.