# CONCLUSION

To solve the problem of insufficient amounts of WSC in the grass ensiling process, the addition of enzymes to release structural carbohydrate was the aim of this study. *Bacillus subtilis* GN 156 was isolated from corn silage and clearly enhanced grass fermentation (Buwjoom, 1998). Therefore, the enzymatic system of *B. subtilis* GN156 was studied and it exhibited the activities of β-1,3-1,4-glucanase, xylanase, dextrinase, CMCase, cellobiase and polygalacturonase, providing relative activities of 100, 12, 12, 1, 1 and 3 %, respectively.

Among several enzymes, β-1,3-1,4-glucanase provided the highest activity against barley β-glucan which contained both 1,3 and 1,4 glycosidic linkages, with low activity to β-1,4 glycosidic linkage in CMC and no activity against β-1,3 glycosidic linkages in larminarin.

According to the substrate on which the enzymes act (Brett and Waldron, 1996), the enzymes of *B. subtilis* GN156 could be classified to 4 groups: hemicellulase (β-1,3-1,4-glucanase and xylanse), cellulose (CMCase and cellobiase), amylolytic enzyme (dextrinase) and pectinase (polygalacturonase). These results show their activities corresponds to the tropical grass components ratio, which comprised 30-40 % hemicellulose, 1-5 % starch and 1-2 % pectin (Stetälä, 1989), except cellulases were low. For complete grass degradation, other source with high cellulose activity may be needed.

Induction of β-1,3-1,4-glucanase production by CM-cellulose, pectin and xylan revealed an increment of activities of 47, 41 and 11-folds, respectively. Maximum β-1,3-1,4-glucanase production was obtained from using 0.8 % (w/v) CMC as an inducer. In addition, the β-1,3-1,4-glucanase showed an optimal pH and temperature of 7 and 60°C, respectively, while stability was wide over a pH of 3-11 and a temperature range of 20-50° C.

Pervaporation was used to concentrate the crude enzyme. Concentration at 37˚C or 55˚C resulted in recovery about 90 %. The concentrated enzyme was therefore used in further studies. Further purification by gel-filtration chromatoghapy using Sephacryl S200 and Sepharose 6B, resulted in poor resolution of β-1,3-1,4-glucanase from other proteins.

Purification of β-1,3-1,4-glucanase was accomplished by using affinity chromatography. Epoxy-activated Sepharose 6B was coupled to β-glucan under the condition of pH 3.0. The β-1,3-1,4-glucanase AJ1 and AJ2 increased in purification of approximately 118-fold with 19.11 % recovery and 823-fold with 9.36 % recovery respectively. The enzymes AJ1 and AJ2 were further purified by using ultrafiltration, with a molecular weight cut off 50,000 dalton filter, resulting in purified J1 and partial purified pJ2. Homogeneity of the enzymes were determined by 10 % SDS – PAGE and 7.5 % native – PAGE. It was concluded that the purified J1 was a single protein approximately of 25 kDa with a pI of 3.5, while the partial purified pJ2 consisted of two proteins of J1 and J2. The purified J1 and J2 had pI values of 3.5 and 3.6, respectively. J2 alone consisted of three-subunit proteins of 40, 32 and 18 kDa. In addition, this is the first report of bacterial β-1,3-1,4-glucanases, which showed the lowest pI of 3.5 and 3.6.

The optimum pH of J1 and pJ2 were 6 and 6.5, respectively, and both were stable in the pH range of 6 - 9 at 4°C for 2 h. The optimum temperature was the same at 60°C. But there was a difference in thermostability. J1 was stable at 20-50°C for 30 min with remaining activity around 50 %. pJ2 was unstable to these temperatures. The remaining activity of pJ2 was decreased to 20 % at 20-40°C and completely inhibited at 50-70°C. Moreover, both J1 and pJ2 β-1,3-1,4-glucanase activities were significantly inhibited by $Mn^{2+}$ ions, and were reduced in the presence of $Cu^{2+}$ ions. In addition, J1 and pJ2 exhibited activities against barley β-glucan, but no activity against CMC, xylan, laminarin and dextrin. Lineweaver – Burk plots for $K_m$ $V_{max}$ and $k_{cat}$ yield the values of 1.53 mg/ml 8,511μU/ml.min and 0.074 $sec^{-1}$, respectively for the purified J1, and values of 4.36 mg/ml, 7,397 μU/ml.min and 0.091 $sec^{-1}$,

respectively for the partial purified J2. Therefore, J1 and pJ2 properties were similar with respect to their optimum pH, pH stability and substrate specificity, but significantly different in optimum temperature, temperature stability and kinetics values indicated that the two β-1,3-1,4-glucanases from *B. subtilis* GN156, J1 and pJ2 were different type.

The degradation patterns from the crude enzyme and J1 action on β-glucan were similar. But they both were different from pJ2 in oligosaccharide obtained. The degradation products from β-glucan hydrolysis by β-1,3-1,4-glucanases from *B. subtilis* GN156 are considered to be triose, tetraose, pentaose and the oligosaccharides larger than cellopentaose. The combination of crude and either J1 or pJ2 could did not enhance both β-glucan and grass degradation activity.

According to the aim of this study, β-1,3-1,4-glucanase involved in grass degradation, therefore its use for increasing the amount of WSC in grass ensiling. In the field of application biotechnology, this enzyme is important in the brewing and animal feedstuff industries (Planas, 2000). β-1,3-1,4-glucanase is an important commercial enzyme. However the study of this enzyme has been limited, due to the cost of substrate, therefore, this study seems to be the first report of β-1,3-1,4-glucanase purification, characterization and degradation patterns with respect to silage.