CHAPTER 4

RESULTS AND DISCUSSION

4.1 Cloning of MBP-*Bs*CsnA

The entire *B. subtilis* CsnA gene was cloned into the pMAL-p5X vector, which had been previously digested with *BamHI* and *XmnI*. The PCR products (~834 bp) were digested with *BamHI-HF* and cloned into corresponding restriction sites on the pMAL-p5X plasmid (Fig. 4.1.a). In addition, the 3-dimensional structure of MBP-*Bs*CsnA was shown in Fig. 4.1.b. This result showed that in the fusion of Maltose Binding Protein (MBP) with the mature enzyme.

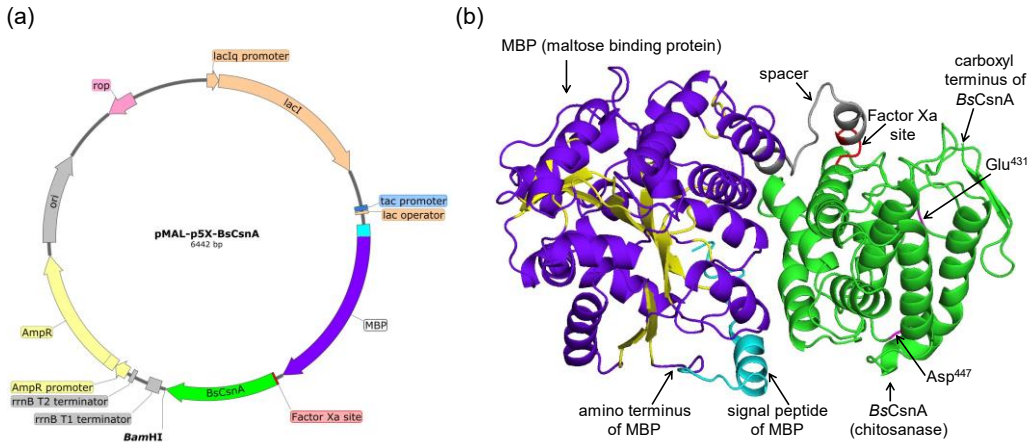

Figure 4.1 Maltose binding protein (MBP)-chitosanase (*Bs*CsnA) construct. (a) MBP-*Bs*CsnA construct in pMAL-p5X vector. (b) 3-dimensional structure of MBP-*Bs*CsnA. The signal peptide of MBP (cyan blue), MBP (violet), β-sheets of MBP (yellow), Factor Xa site (red) and *Bs*CsnA (green) were shown. The two catalytic residues of *Bs*CsnA (Glu^{431} and Asp^{447}) were magenta-coloured.

4.2 Expression of MBP-*Bs*CsnA in *E. coli* system

The recombinant MBP-*Bs*CsnA was expressed in the *E. coli* TOP10. The enzyme was collected at different times after induction and extracted from different compartments. Two hundred fifty milliliters of recombinant MBP-*Bs*CsnA were grown in Rich-Glucose-Amp medium until OD_{600} reached ~0.5 before IPTG was added to a final concentration of 0.3 mM, and incubation continued at room temperature (~ 27°C). Fifty ml of samples were taken at 0, 2, 4, and 20 h after induction with IPTG and extracted the enzyme from culture medium (broth), periplasm, and cell lysate.

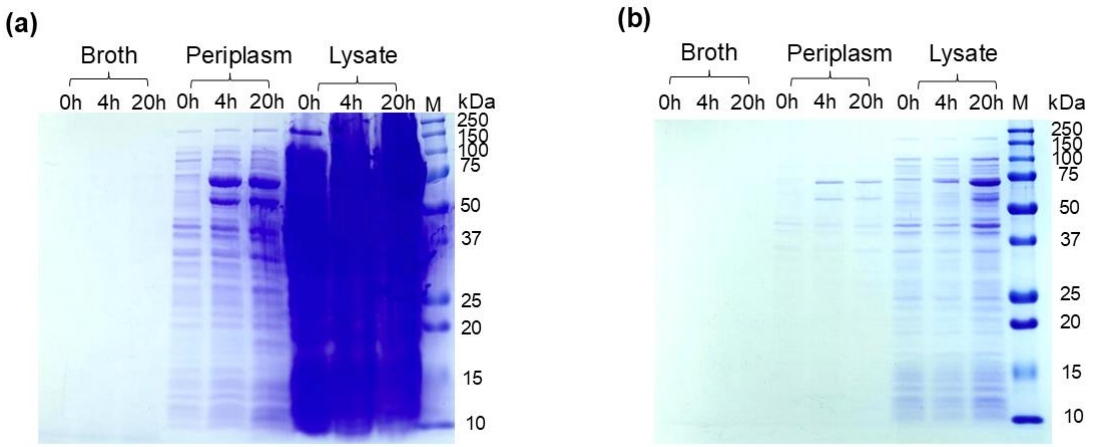

Figure 4.2 SDS-PAGE analysis of recombinant MBP-BsCsnA from different compartments of *E. coli* TOP10. The broth, periplasm, and lysate were loaded approximately 10 µl and 3 ug as indicated in panel (a) and (b), respectively. The gel was stained with Coomassie brilliant blue and All blue Prestained Protein Standards (BioRad #1610373, U.S.A.) was used as a marker.

The SDS-PAGE of the recombinant *Bs*CsnA in all three compartments was shown in Figure 4.2. The SDS-PAGE analysis of secreted enzyme into broth after inducing for different time was shown in Figure 4.2.1. The enzyme from MBP-*Bs*CsnA was secreted into the culture supernatant after inducing for 2h and getting increase for

20h. The molecular weight is approximately 73 kDa from SDS-PAGE analysis. The result demonstrated that the enzyme can be secreted into crude supernatant (broth).

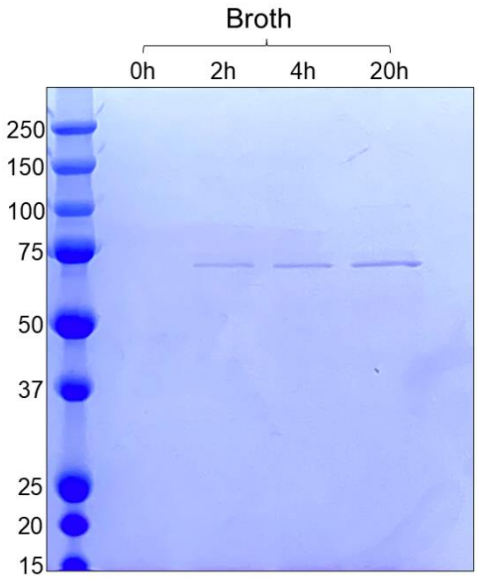

Figure 4.2.1 SDS-PAGE analysis of recombinant MBP-*Bs*CsnA from broth at different time after induction with 0.3 mM IPTG. Approximately 10 µl of broth were loaded into each lane. The gel was stained with Coomassie brilliant blue and All blue Prestained Protein Standards (BioRad #1610373, U.S.A.) was used as a marker.

4.3 Comparison of the bacteria cell growth in Terrific Broth (TB), Lurria Broth (LB), and Rich media

The condition of optimization was optimized by growing the recombinant MBP-*Bs*CsnA in different growth media. The single colony was picked and incubated on Terrific Broth (TB), Luria Broth (LB), and Rich media, respectively. After the incubation of bacteria cells on TB, LB, and Rich media at 37°C for 15 hours with 250rpm shaking, the appearance on TB media was turbid whereas on LB and Rich media was clear. The results showed that bacteria cells can grow on TB media and cannot grow neither LB

nor Rich media. TB media contains many chemical ingredients compared to LB and Rich media. KH_2PO_4 and K_2HPO_4 are additional chemicals on TB media.

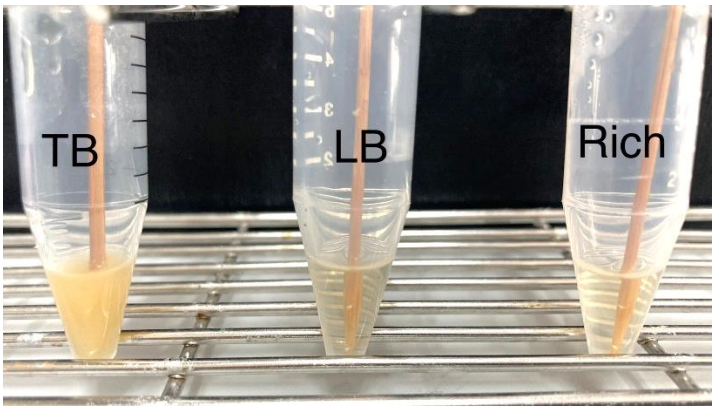

Figure 4.3 Comparison of the recombinant MBP-*Bs*CsnA in different bacteria growth media. The analysis is based on the turbidity of growth media.

Table 4.3 Comparison of chemical ingredients and the percentage of bacteria growth media

Components	TB (%)	LB (%)	Rich (%)
Tryptone	1.2	10	10
Yeast	2.4	0.5	0.5
NaCl	-	0.5	0.5
Glucose	-	-	0.2
Glycerol	0.5	-	-
KH_2PO_4	0.23	-	-
K_2HPO_4	1.25	-	-

4.4 Optimization, expression and purification of MBP-*Bs*CsnA

The recombinant MBP-*Bs*CsnA was successfully expressed in *E. coli* TOP10 and purified by one step purification using amylose beads. We optimized the conditions for enzyme expression and found that TB-Amp media is the optimal media to inoculate the recombinant MBP-*Bs*CsnA and the enzyme induction with 0.3mM IPTG at 27°C for 20hr can produce the maximum level of active enzyme. Cells were cultured in 100ml shaken flask and the recombinant enzyme was purified from culture supernatant. The enzyme was purified by amylose beads. The result indicated that the enzyme could be expressed in *E. coli* TOP10 as demonstrated by SDS-PAGE and zymogram analysis, respectively (Figure 4.4 and Figure 4.4.1).

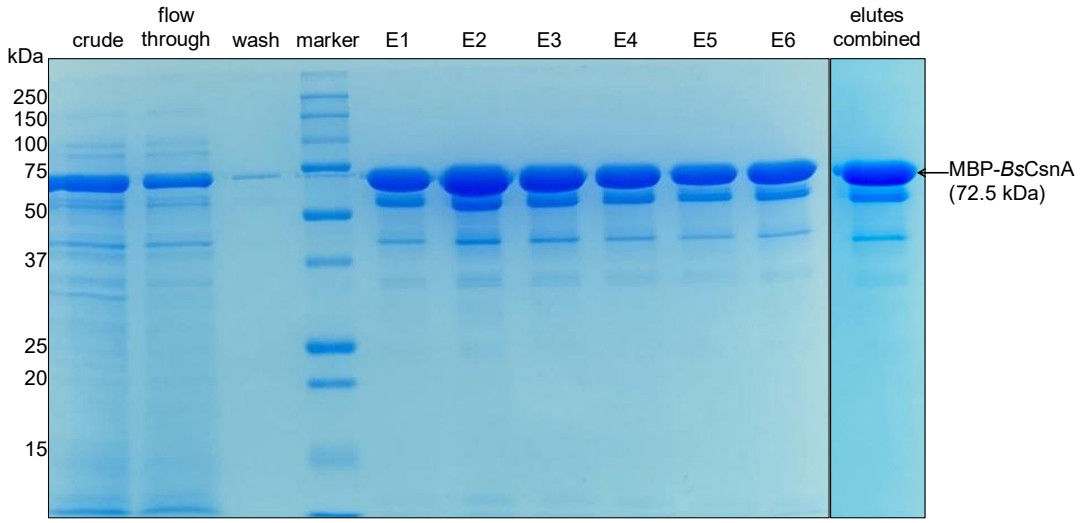

Figure 4.4 Expression and purification of recombinant MBP-*Bs*CsnA. SDS-PAGE analysis of MBP-*Bs*CsnA purified from crude supernatant. About 20 μL of crude, flow through, wash, elutes (E1-E6) and elutes-combined were loaded as indicated. MBP-*Bs*CsnA was seen at the expected size (72.5 kDa). The gel was stained with Coomassie brilliant blue and All blue Prestained Protein Standards (BioRad #1610373, U.S.A.) was used as a marker.

The SDS-PAGE analysis confirmed the extracellular expression of the enzymes MBP-*Bs*CsnA (Fig. 4.4). The molecular weight of MBP-*Bs*CsnA was predicted to be 72.5 kDa, which consists of 42.5 kDa of MBP and 30 kDa of *Bs*CsnA. Chitosanase activity of the purified recombinant enzyme was shown by in-gel activity staining (Figure 4.4.1) as well as by standard chitosanase assay. The specific activity of purified enzyme was 400 U/mg. The purification of MBP-*Bs*CsnA and His-*Bs*CsnA was summarized in Table 4.4.

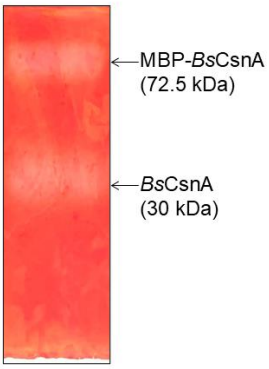

Figure 4.4.1 Zymogram analysis of purified recombinant MBP-*Bs*CsnA by transfer gel-zymography. Mixed protein MBP-*Bs*CsnA with loading buffer (Laemmli buffer) without reducing agent and heating. The protein gel was laid on top of a polyacrylamide gel, containing 0.1% chitosan (low molecular weight). The gel was stained with 2% Congo Red. White band indicated chitosanase activity.

This study presents an interesting approach to express and characterize a fusion protein, MBP-*Bs*CsnA, in *E. coli* TOP10. The gene encoding the *Bacillus* chitosanase can be fused successfully with Maltose Binding Protein using pMAL vector. Recombinant chitosanase with maltose binding protein fusion can be expressed and purified successfully using pMAL protein fusion and purification system. It means that pMAL vector, protein fusion, and purification system have ability to clone, express, and purify *Bs*CsnA. Therefore, *Bs*CsnA can be secreted efficiently by *E. coli* expression system.

Table 4.4. Comparison of purified MBP-*Bs*CsnA and His-*Bs*CsnA

Purified enzyme	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Moles of protein (nmol)	Specific activity (U/nmol)
MBP-*Bs*CsnA	3.15	1236	400	13.7	28.64
His-*Bs*CsnA	5.6	5063.2	900	33.33	27.13

When estimated from the specific activity of the purified enzyme (400U/mg), about 150000 U of crude enzyme can be obtained from a 1-L culture in a shake flask, indicating the enzymatic efficiency of the recombinant chitosanase fusion protein. However, it is noted that this specific activity of the purified enzyme is lower than previously reported His-tagged fusion with *Bs*CsnA, which had a specific activity of 900U/mg (Pechsrichuang et al., 2013). This discrepancy could arise from differences in fusion tags and purification methods.

4.5 Effect of temperature and pH on MBP-*Bs*CsnA

The optimal temperature for recombinant enzyme activity was 55°C under standard assay conditions (Fig. 3a, purple solid line). The enzyme was stable up to 50°C after incubation for 30 min at pH 6.0, without substrate. Less than 10% of residual activity could be detected after incubation at 60°C under these conditions (Fig. 3a, dashed line). The optimal pH of recombinant enzyme was 6.0 (Fig. 3b, solid line). Notably, the chitosanase activity was stable within pH 2-9 after incubation at 30 °C for 24 h (Fig. 3b, dashed line).

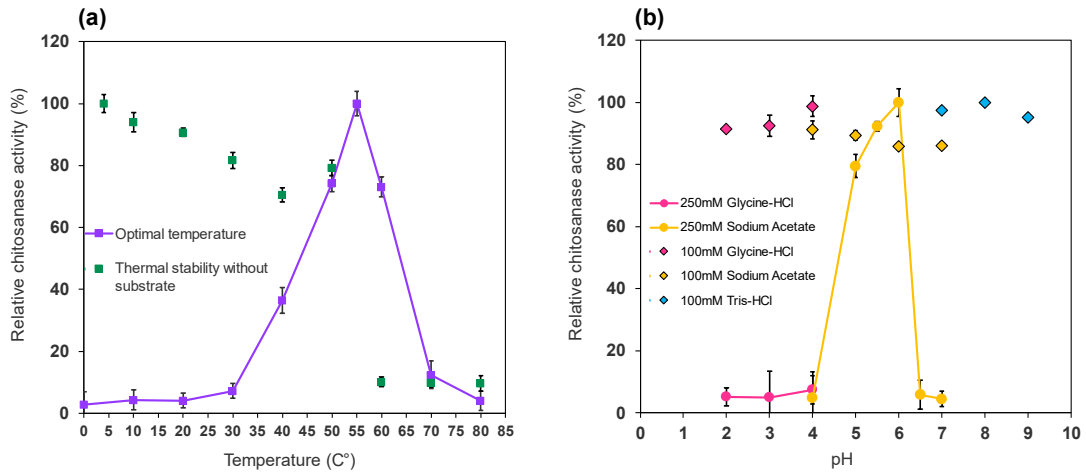

Figure 4.5 Effect of temperature and pH on MBP-chitosanase activity. (a) The optimal temperature for MBP-*Bs*CsnA (purple solid line) was determined using 0.5% chitosan (practical grade) in 200 mM sodium acetate buffer (pH 5.5) at indicated temperatures for 5 min incubation. The chitosanase activity (%) compared to the highest activity within the temperature range was plotted on the x-axis and the temperature values on the y-axis. The effect of temperature on stability of MBP-*Bs*CsnA in the absence of chitosan substrate was tested using 50 mM sodium acetate buffer (pH 6.0) for 30 min, using the standard assay. The chitosanase activity (%) compared to the activity at storage temperature (4°C) was plotted on the x-axis and the temperature values on the y-axis. Each dot represented the average of triplicates from two independent experiments and the error bars represented the ratio standard deviation of the mean. (b) The optimal pH for MBP-*Bs*CsnA was determined using 250mM glycine-HCl buffer (pink solid line) and 250mM sodium-acetate buffer (yellow solid line) at 50°C incubation. The effect of pH on stability of MBP-*Bs*CsnA was tested at pH 2-4 using 100mM glycine-HCl buffer (pink dotted line), pH 4-7 using 100mM sodium-acetate buffer (yellow dotted line) and pH 7-9 using 100mM Tris-HCl buffer (blue dotted line) at 50°C incubation. The relative chitosanase

activity (%) was plotted on the x-axis and the pH values on the y-axis. Each dot represented the average of triplicates from two independent experiments and the error bars represented the ratio standard deviation of the mean.

When the enzyme was incubated for 30 min at 50°C and pH 5.5, in the presence of chitosan, the residual activities of the enzymes were less than 100%. These results indicated that substrates could prevent thermal inactivation of the chitosanase activity. In addition, the thermal inactivation kinetics at 50°C showed that the enzyme was more stable in the presence and absence of 0.5% chitosan (low molecular weight) than *Bs*CsnA-10xHistidine tag, as shown in Fig. 4.5.1.

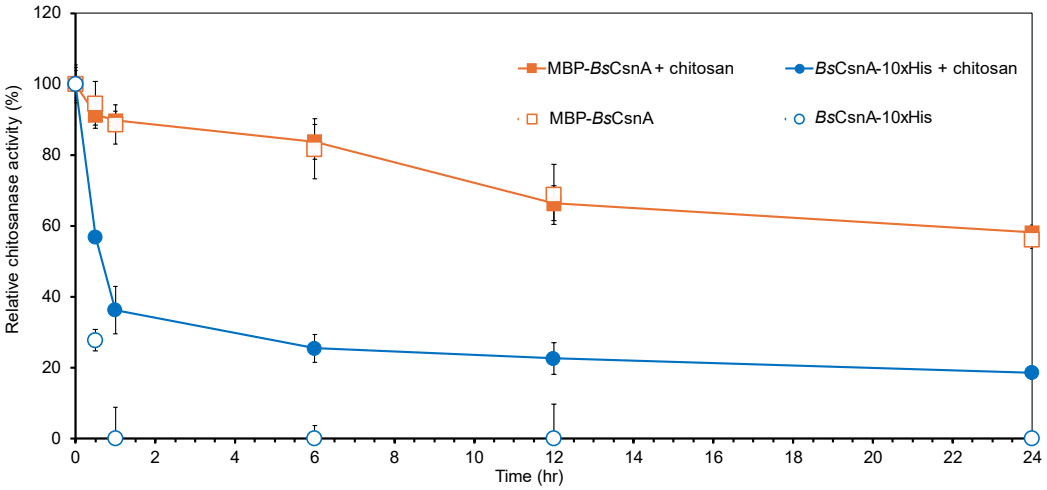

Figure 4.5.1 Thermostability of MBP-*Bs*CsnA in the presence and absence of chitosan substrate. Thermostability of MBP-*Bs*CsnA at 50°C in the presence (orange square, solid line) or the absence (orange square, dashed line) of 0.5% low molecular weight chitosan was measured at the indicated temperatures. Thermostability of *Bs*CsnA-10xHistidine tag in the presence (blue circle, solid line) or the absence (blue circle, dashed line) of chitosan was used as a control. Each dot represented the average of triplicates, and the error bars represented the ratio standard deviation of the mean.

In this study we show that the properties of MBP-*Bs*CsnA enzyme is similar to those of previously reported His-tagged fusion (Pechsrichuang et al., 2013), except that thermostability was significantly improved. The chitosanase activity was stable in the widest range of pH. It was not possible to determine the enzyme activity at pH > 7.0 because the chitosan substrates were insoluble at this condition. It is similar to those of previously reported His-tagged fusion (Pechsrichuang et al., 2013) while the thermostability was significantly improved. The enhancement of chitosanase thermostability by substrate was confirmed by thermal inactivation experiment. The thermal inactivation kinetics at 50 °C showed that the enzyme was more stable in the presence and absence of 0.5% chitosan (low molecular weight) than *Bs*CsnA-10xHis-tagged fusion. These results indicated that the recombinant MBP-*B. subtilis* chitosanase could be attractive for industrial applications because it is relatively thermo- and pH-stable.

4.6 Chito-oligosaccharide production using MBP-*Bs*CsnA

The hydrolysis reaction mixture was analyzed by thin layer chromatography (TLC). The TLC results (Fig. 5) showed that after 6 hours, Mor-CHOS and MB-CHOS, primarily DP3, DP4, and DP5, became detectable. After 48 hours of hydrolysis, Mor-CHOS and MB-CHOS were made up mainly of dimers and trimers. These results suggested that the MBP-*B. subtilis* chitosanase can cleave GlcN–GlcN links, which is common to all known chitosanases.

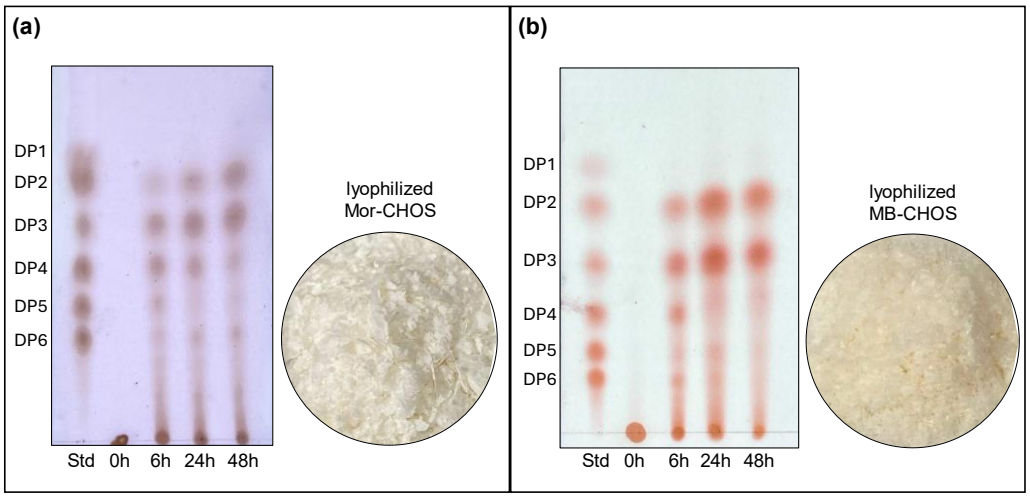

Figure 4.6 Thin layer chromatography analysis of chitosan hydrolyzed by MBP-*Bs*CsnA. The hydrolytic products of (a) 500-600 kDa Morena-chitosan and (b) Marine Bioresource-chitosan at indicated time points were analyzed by TLC. A mixture of 10 mM (DP1-DP2) and 5 mM (DP3-DP6) chitosan-oligosaccharides was used as a standard (Std). The lyophilized hydrolytic products of Morena-chitosan (Mor-CHOS) and that of the Marine Bioresource-chitosan (MB-CHOS) were also shown.

The findings from TLC analysis of chitosan hydrolysis by MBP-*Bs*CsnA provide valuable insights into the substrate specificity and catalytic mechanism of the enzyme. The conversion of chitosan hexamer into smaller oligomers suggests that the MBP-*Bs*CsnA has broad substrate specificity and exhibits endo-type hydrolytic activity by cleaving internal glycosidic linkages within chitosan chain. The MBP-*B. subtilis* chitosanase can cleave GlcN–GlcN links, which is common to all known chitosanases.

4.7 Anti-inflammatory activity of Chito-oligosaccharide in human macrophage cell

The results showed that Mor-CHOS at 25 and 50 ug/mL (Fig. 7a), and MB-CHOS at 100 µg/mL (Fig. 7b) significantly decreased the LPS-induced IL-1β release from THP-

1 monocytes ($p < 0.001$) in a dose-dependent manner. At the highest concentration (200 μg/mL) from both CHOS showed that it could stimulate IL-1β secretion. The maximum inhibitory effect obtained with these CHOS was comparable to the effect of 0.2 μg/ml (0.5 μM) dexamethasone.

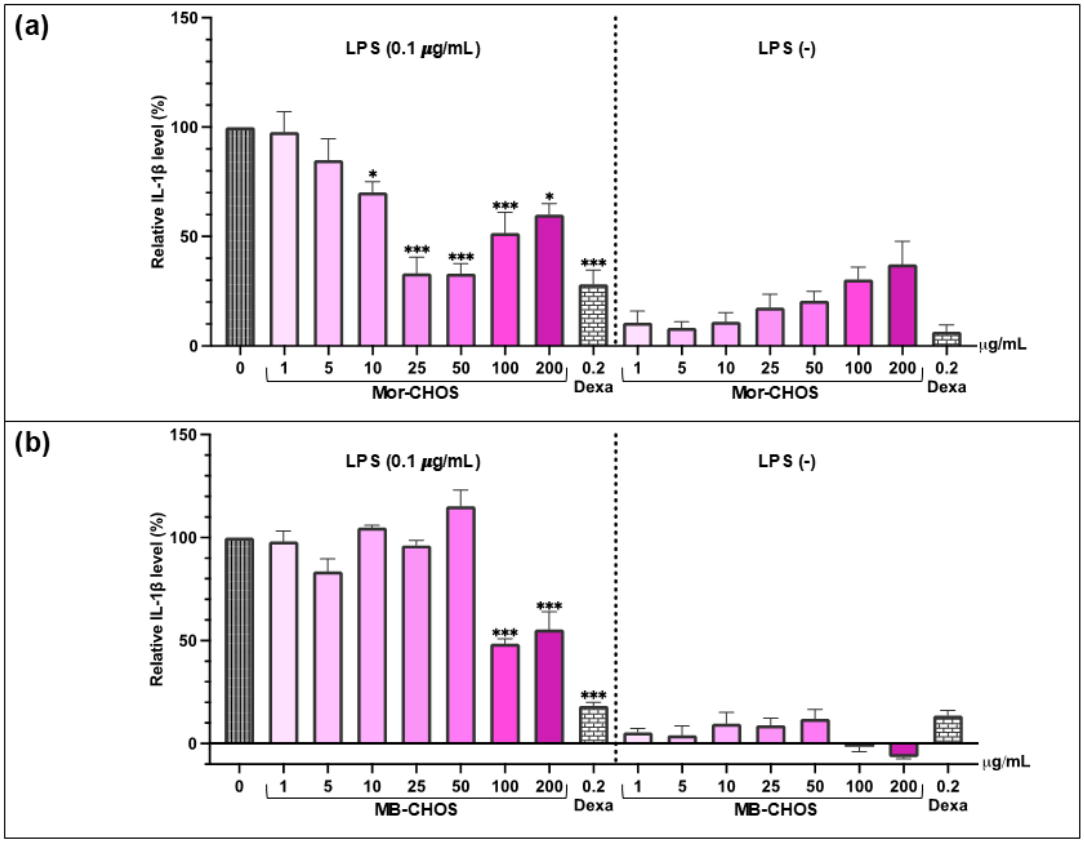

Figure 4.7 IL-1β response in vitD3-differentiated THP-1 monocytes pretreated with Mor-CHOS/MB-CHOS (1 - 200 μg/mL). The IL-1β response of THP-1 cells was compared between cells without pretreatment and (a) Mor-CHOS pretreated cells or (b) MB-CHOS pretreated cells. Dexamethasone (0.2 μg/mL) was used as a positive control. Each column represented the average value of triplicates from two independent experiments and the error bars represented the standard deviation of the mean. Statistical analysis was performed using Ordinary One-way ANOVA, Dunnett's multiple comparisons test with a single pooled variance and P value style: *P < 0.033, **P < 0.002, ***P < 0.001.

Chito-oligosaccharide, produced by bioconversion 1% chitosan dissolved in mild acid using MBP-*Bs*CsnA, showed anti-inflammatory activity. Mor-CHOS 25 and 50 μg/mL gave the best anti-inflammatory effect than MB-CHOS 100 μg/mL. At the highest tested concentration (200 μg/mL), the CHOS might stimulate inflammation in human THP-1 macrophages. The results indicated that the immunomodulatory property of CHOS depended on the chitosan source which could lead to biological effects on the human cells.

4.8 Production of cream

Since MB-CHOS has an anti-inflammation effect, it was used as an active ingredient in skincare products, especially cream. There were 2 variants of cream, cream with 100 ug/ml MB-CHOS and vanilla fragrance (Figure Xa) and cream with 100 ug/ml MB-CHOS, vanilla fragrance, and curcumin (Figure Xb). The cream was produced not only to help reduce inflammation of the skin but also to keep the skin healthy and moist. However, clinical trial is recommended to do for further test.

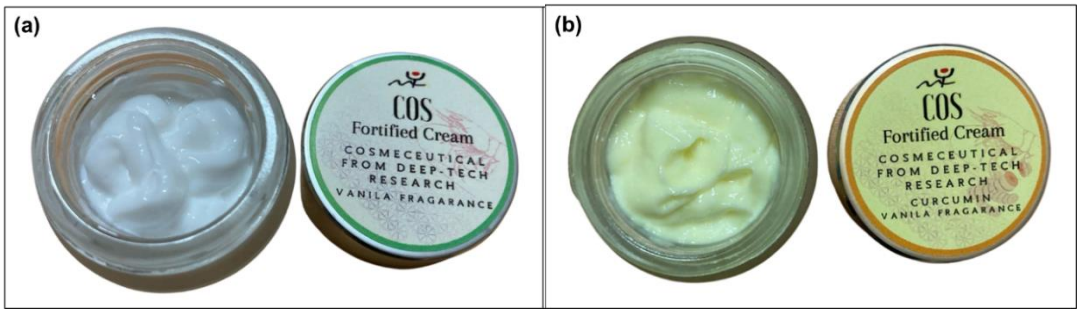

Figure 4.8 Cream products contain 100ug/ml of MB-CHOS with vanilla fragrance (a), and 100ug/ml of COS with vanilla fragrance and curcumin (b).