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Production and Separation of Chitooligosaccharides using *Capsicum annuun* Immobilized Chitosanase

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ABSTRACT

The products of chitosan hydrolysis are chitooligosaccharides and are used mainly for medical applications due to their specific biological activities. The objective of this study was to prepare and separate chitooligosaccharides (COS_N)from chitosan using *Capsicum annuum* immobilized chitosanase. We firstly studied the optimal reaction conditions, in order to maximizethe production of COS_N by using immobilized chitosanase. The optimal temperature and pH for COS_N production were determined to be 55°C and pH 5.6. The ratio of enzyme /substrate should not be lower than 0.95 U/mg in reaction mixture for maximal COS_N production. From the time course of the enzymatic degradation of soluble chitosan, it was found that the hydrolysis rate was fast during the first 1.5 h. In the following reaction time, no significant change occurred. The immobilized chitosanase could be recovered and reused repeatedly for four times with high COS_N production. Relative COS_N production at optimum hydrolysis conditions was 55.21 %.The prepared $COS_N$was separated by ultrafiltration to four fraction (I, II, III and IV) according to the molecular weights (>100, 100-10, 10-1 and <1KDa, respectively). The total yield recovery after separation by ultrafiltration was 100%. Fraction II and III with COS_N yield of 33.5 % and 31.3 % and COS ratio of 18.31 % and 49.72%, respectively were higher than that of fraction I and IV.

Keywords: Chitooligosaccharides production, chitosan, Immobilized chitosanase, Optimization, T.L.C., Sephadex-G-25 and Ultrafiltration.

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INTRODUCTION

Chitosan is a deacetylated derivative of chitin which is a linear polysaccharide consisting of β-1,4 N-acetyl-glucosamine [1]. Chitosan is insoluble in water, but dissolves in aqueous solutions of organic acids, such as acetic, formic, and citric acids, and in inorganic acids, such as diluted hydrochloric acid. Chitooligosaccharides (COS) are the hydrolysate of chitosan mainly made up of β-1,4 linked D-glucosamine (GlcN) and partially of β-1,4 linked N-acetyl-D-glucosamine (GlcNAc). Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the β-1, 4 glycosidic bond hydrolysis of chitosan to produce chitooligosaccharides.

Large amount of COS can be obtained by acidic hydrolysis, oxidative degradation, microwave, $^{\gamma}$-irradiation or by enzymatic hydrolysis [2-5]. Chemical hydrolysis is performed at high temperatures under highly acidic conditions and produced a large amount of glucosamine (chitosan monomer), owing to difficulties in controlling the progress of reaction.

Chitooligosaccharides can be obtained from the partial hydrolysis of chitosan. As the higher oligosaccharides such as pentamers and hexamers are intermediate products in chitosan hydrolysis, it is necessary to control the hydrolysis reaction to produce them at high yield. Utilizing enzymes in an immobilized form would be effective to prepare mixture higher oligosaccharides for the following reasons: (1) as enzymes immobilized on insoluble materials can be separated immediately from the reaction mixture and hydrolysis reactions can be terminated easily (2) the reaction can be stopped without any chemicals or heating; (3) enzyme catalysts can be recovered and used repeatedly [6]. Several prior studies described separation of COS. Capillary electrophoresis was employed to separate and monitor COS with two to six saccharides chains [7]. Size exclusion chromatography (gel filtration) was applied to realize the separation of COS [8]. However, these methods need expensive equipment and do not allow for large-scale preparation of COS with narrow DP. Several COSs with single DP were separated by immobilized metal affinity column chromatography but the tetramer was the upper limit of efficient separation in this system [9]. Ultrafiltration membrane was applied to separation COS with different molecular weight [10-11].Several studies showed that COS possessed a huge potential in medicine and food fields due to its wide bioactivity, such as antitumor activity, radical scavenging, antimicrobial activity, immunity modulatory effect and wound healing [12-14].All the techniques applied to prepare COS produce the mixtures containing COS with different molecular weights. Most of reported bioactivities of COS were also assayed using mixtures. It is difficult to know which COSs with well-defined degree of polymerization (DP) play a significant role in the bioactivity assay. For further bioactivity studies, the separation of COS with very narrow molecular weight was required.

In previous work, we immobilized *Capcicum annuum*chitosanaseIC_N andisoenzyme chitosanases (IC_A and IC_B) on chitin by covalent binding with high activity and storage stability [15]. In the present study, chitooligosaccharides (COS) with different molecular weight were prepared from chitosan by *Capsicum annuum* immobilized chitosanase. They were separated according to their molecular weight by using ultrafiltration technique.

MATERIALS AND METHODS

Materials:

Chitosan with molecular weight 100-300 KDa and 70-85% deacetylated, chitin, glucosamine, dimers, tetramers andSephadex G-25 were purchased from Merck chemical Co . All other reagents used were of highest quality available. Immobilized chitosanases IC_N, IC_A and IC_B by covalent binding were prepared from fresh *Capcicum annum* leaves as described previously [15].

Methods:

Preparation of chitosan solution

Soluble chitosan for determination chitosanase activity and production of chitooligosaccharides and low molecular weight chitosan was prepared as follow: ten grams of chitosan powder was suspended in 400 ml distilled water and dissolved while being stirred in 5ml concentrated acetic acid. This solution was made with up to 1 L of water, and the pH was adjusted by using 1N NaOH [16].

Measurement of immobilized chitosanase activity

The reaction mixture of 0.9 ml of 1% soluble chitosan dissolved in 0.05 M sodium acetate buffer, pH 5.8, adequate amount of immobilized chitosanase and 1 ml of 0.05 M sodium acetate buffer, pH 5.8was incubated at 40°C for 1.5 h. The reaction was stopped by boiling in water bath for 10 min. The mixture was centrifuged at 3000 rpm for 15 min. The concentration of reducing sugars produced from chitosan was measured by dinitrosalicyclic acid method (DNS) [17] using glucosamine as standard.

One unit of chitosanase was defined as the amount of enzyme that could liberate one µmole of reducing sugar under the standard assay conditions using glucosamine as standard. The activity of chitosanase value was average values of three repeated measurements.

Preparation of immobilized chitosanases

One g chitin was shaken in 5ml 0.1M HCl containing 5% (v/v) glutraldehyde (GA) for 24 h at 30°C. The solubilized chitosan was precipitated by addition of one ml of 0.1M NaOH. The precipitates were collected by filtration and washed with distilled water to remove the excess GA. The wet chitin was mixed with 2.0 ml of enzyme solution (400 U free chitosanases C_N or$C_A$or C_B). After being shaken for 1.0 hat 30°C, the unbound enzyme was removed by washing with distilled water. The immobilized chitosanase activity was determinedaccording to the method of El-Sayed *et al*. [15].

Chitooligosaccharides (COS_N) production by immobilized chitosanases (IC_N) from chitosan

Production of chitooligosaccharidesCOS_N was accomplished through enzymatic hydrolysis of chitosan using immobilized chitosanase (IC_N). The reaction mixture containimmobilized chitosanase(IC_N) chitosan ratio 0.95 U/mg in acetate buffer pH 5.6.It was incubated at 55°C for 1.5 h.Then, the reaction mixturewas centrifuged and the supernatant were taken asCOS_N.

Measurements of chitooligosaccharides production

- Enzyme substrate ratio (U/mg) = Immobilized chitosanase activity (U) / Chitosan (mg)
- Concentrations of the prepared COS_S were estimated by dinitrosalicylic acid method [17] and calculated as mg glucosamine.
- Relative COS_S production (%) = $\frac{\text{Chitooligosaccharides concentration (mg)}}{\text{Chitosan concentration (mg)}} \times 100$
- COS_SRatio % = COS (mg) / COS dry weight (mg) ×100

Optimum condition for chitooligosaccharides production

- **Dependence of COS production on different incubation pH's and times.**

The amount of chitooligosaccharides produced from chitosan by the prepared immobilized chitosanase IC_N were determined at different incubation pH ranged from 4.5 to 8.0 and different time intervals up to 21h.

- **Effect of different temperatures and thermal stability on chitooligosaccharides production.**

The amount of chitooligosaccharides produced from chitosan by the prepared immobilized chitosanase IC_N were determined at different incubation temperatures ranged from 30-70°C. For measurement of thermo-stability, immobilized enzyme was preincubated at the various temperature (40-70°C) for one hour before preparation chitooligosaccharides.

- **Changes on COS production at various enzyme substrate ratios.**

The amount of chitooligosaccharides produced from chitosan by the prepared immobilized chitosanase IC_N were determined at different enzyme substrate ratios ranged from 0.19 to 1.9 U/mg. Enzyme substrate ratios were calculated by immobilized enzyme activity per mg chitosan weight (U/mg).

- **Change in the COS productionfollowing serialaddition of new chitosan at optimum conditions.**

Production of chitooligosaccharides (COS_N, COS_A, and COS_B) were accomplished through enzymatic hydrolysis of chitosan using immobilized chitosanases (IC_N, IC_A and IC_B) at optimum standard conditions.Immobilized chitosanase was added to 10 ml 1% chitosan solution at ratio of enzyme : substrate 0.95 U/mg in a water bath and 50^{o}C for 1.5 h at pH 5.6. At the end of the reaction time, the immobilized chitosanase was collected and washed with distilled water and resuspened in 10 ml of freshly prepared substrate to start a new run. This step was repeated for 10 times. Chitooligosaccharides concentration in the supernatants were determined after each run.

Separation of chitooligosaccharides (COS_S**).**

COS_N, COS_A, and COS_B prepared after four runs at optimum conditions were collected and stored at 4^{o}C till used.

Gel filtration.

The prepared COS_N, COS_A and COS_B from chitosan by immobilized enzymes (IC_N, IC_A and IC_B, respectively)were concentrated by lyophilization. Sephadex G-25 powder was previously soaked in distilled water for two days. Fine particles must be removed by decantation. Sephadex G-25 column (1.0 × 37 cm)was equilibrated with 0.01M acetate buffer, pH 5.8 before used. Dry COSs (COS_N, COS_A and COS_B) were applied to the top of the column, and the COS were eluted with the same buffer at a flow rate of 15ml/h. Four fraction at tube 4th, 5th, 6th and 7th were collected and lyophilized. COS concentrationin each fraction was determined by DNS[17]. Weights of thedry COS_A, COS_B and COS_N in each fraction were determined.

Ultrafiltration of chitooligosaccharides

The prepared COS_Nsolution was fractionated by successive steps of tangential flow filtration in a Cross flow filtration system (Sartorius sartoflow) using filters with a gradual reduction on the cut off (MWCO100,000, 10,000 and 1,000 Daltons membranes, Sartorius, polysulfone).COS I (>100 KDa) was the low chitosan molecular weightnot passed out through MWCO 100.0 kDa membrane; COS II(100.0-10.0 kDa) was the COSspassed out through MWCO 100.0 kDa membrane, but not through 10.0 kDa; COS III (10.0-1.0 kDa) was the COSs passed out through MWCO 10.0 kDa membrane, but not through 1.0 kDa and COS IV (<1.0 kDa) was the COSs passed out through 1.0 kDa membrane. About 250 ml concentrate (retentate) was collected from each step yielded fractions I, II and III. The permeate fraction (IV) was collected at the end of the filtration process. All fractions were lyophilized and stored at - 4ºC.

Qualitativeanalysis of chitooligosaccharides

Detection of chitooligosaccharides in each fraction (4th, 5th, 6th and 7th) collected from the Sephadex G-25 column and in fraction (I, II, III and IV) collected by ultrafiltration were conducted by thin layerchromatography (TLC) using aluminium sheet of silica layer. COS_N, COS_A and $COS_B$and standard (glucosamine) were subjected to one dimensional chromatography on aluminium sheet of silica layer. They were dissolved in deionized water. One microliter of each sample solution was applied to the chromatoplate with micropipette. The chitooligosaccharides were developed with a solvent system of n-propanol: water: concentrated ammonia (7:2:1). Sugar spots on the plates were visualized by charring with 10% H2SO4 in ethanol and each spot was identified [18].

Statistical analysis

Data are expressed as the mean ± standard error (SE) from at least three experiments.

RESULTS AND DISCUSSION

Attention in the chitosan field has recently been concentrated in the production of useful chitooligosaccharides (COSs) because oligosaccharides are not only water soluble but also possesses versatile functional properties. The most important tool in the biodegradation of chitosan to its oligosaccharides (COS)

is chitosanolytic enzymes. In the present study, immobilized (*Capsicum annuum*) chitosanases (IC_N, IC_A and IC_B)previously prepared [15] were chosen for production of chitooligosaccharides (COS_N, COS_A, and COS_B). The concentration of COS (mg), relative COS production(%) and COS ratio (%) were determined.

Optimum conditions for production of chitooligosaccharides (COS_N) by chitosanase IC_N.

For maximal production of COS_N from chitosan, optimal conditions including pH, temperature, thermotability, incubation time, enzyme substrate ratio and reusability of the immobilized enzyme were investigated.

1. **Chitooligosaccharides (COS_N) production at different pH's.**

To investigate the effect of different pH's on COS_N production enzyme/chitosan ratio 0.95 U/mg was incubated at 40°C for 1.5 h in a total volume 2 ml. The pH values in the reaction ranged from 4.5 to 8.0. It was found that the production of COS_Nincreased gradually up to pH 5.6 and above pH 5.8COS_Nproduction decreased(Figure 1).

2. **Effects of temperature and thermostability on the COS_N production.**

To understand the effect of temperature on COS_N production, the enzyme reaction was carried out at various temperatures (40-70 °C) for 1.5 h. The results showed that the highest relative production of COS_N was at widerange temperature from 40 to 60°C with maximum COS production at 55°C (Figure 2).

The effect of pre-incubation of the enzyme at different temperatures for 1.0 h on COS_N production was also studied. As shown in fig (2), the enzyme was stable below 50°Cfor 1.0 h preheated. Only 16% of COS production at 50°C preheated was decreased.

3. **Chitooligosaccharides COS_N production at different times.**

Figure (3) showed that the time course of COS_N production during 21 h at 55°C. The COS_N production increased with increasing time during the first 1.5 h, while during the following reaction time, no significant increase in COS_N production was occurred, suggesting that the enzyme was an endo type enzyme [19].

4. **Change chitooligosaccharides COS_N production at various enzyme/substrate ratio.**

COS_N production increasedrapidly with increase the enzyme substrate ratio from 0.38 to 0.95 U/mg. More enzyme added in the reaction solution (above 0.95 U/mg) led to slow increase in COS_N production (Figure 4). Kuo *et al.*[20] and Gao*et al.*[19] reported that the 0.2 U/mg chitosan was used for high hydrolysis of chitosan.

5. **Production of chitooligosaccharides by serial addition of new chitosan.**

Production of COS_N, COS_A and $COS_B$by repeated use of the immobilized enzymes IC_A, IC_B and $IC_N$for 10 run under the optimum conditions of our experimentswas showed in figure (5). COSsproduction during 10 run decreased gradually. The gradual decrease in COS production may be attribute to the strong bound between enzyme and chitin by covalent binding with glutraldehyde cross linking which lead to high stability of the immobilized enzyme during reusing process [15].The results also may be explained by the high thermostability of the immobilized enzyme at 40°C as mention above.

Fractionation of COS by using Sephadex G-25

Production of chitooligosaccharides(COSs) were prepared at optimum conditions (pH 5.6, 55°C with enzyme substrate ratio 0.95 U/mg for 1.5h). COSs were labelled as COS_N, COS_A and COS_B that produced by immobilized chitosanase IC_N, IC_A and IC_B, respectively. COSs were collected by centrifugation and lyophilized. The COSs were fractionation by Sephadex G-25 to four fractions (4^{th}, 5^{th}, 6^{th} and 7^{th}) figure (6). The total yield

of COS_N produced from chitosan was higher than of $COS_A$and COS_B. On the other hand, the total COS ratio % was higher in COS_N (16.5 %) than in COS_A and COS_B(8.82 and 8.58 %) Figure (7).There were high difference in the COS_N ratio % of fractions number 6th and 7th(16.5and 17.45 %, respectively) than in fraction 4th and 5th (5.2 and 2.87 %).

Separation of chitooligosacharides (COS_N) by using ultrafiltration technique

COS_N was chosen for our further studies because the yield of COS_N was more than of COS_A and COS_B. We maximized the yield of COS_N by recycling the incubation of immobilized chitosanase IC_N for four repetitions. High amount of $COS_N$was prepared in short time. Chitooligosaccharides (2650mg) in 3650 ml of hydrolysate from 4800 mg chitosan with relative production value 55.2% were prepared. They were used for separation of chitooligosaccharides by ultrafiltration technique.

Four different kinds of chitooligosaccharides with different molecular weightswere separated from COS_N by ultrafiltration. They were labelled as COS I (>100KDa), COS II (100-10KDa), COS III (10 - 1 KDa) and COS IV (< 1. KDa). All fractions were collected and lyophilized with the yield of COS I (505 mg),COS II (900 mg), COS III (840 mg) and COS IV (442 mg)as shown in table (1). The COS ratio %of COS II and COSIII (18.31 and 49.72%, respectively) were higher than in COSI and IV (6.76 and 5.1%, respectively). These mean that fractions of COS II and COSIII contain high amount of COS than of COS I and COS IV. These results gave a good evidence that ultrafiltration membrane techniques was successful method for separation. Jung *et al.*[21] used ultrafiltration membrane for separation of COS to four fractions. They used the ultrafiltration membranes of MWCOS 30.0, 10.0 and 5.0 kDa. The yields of COS I, COS II, COS III and COS IV fractions were 16.1 %, 19.37 %, 35.84 % and 28.68 %, respectively.

Identification of the chitooligosaccharides fraction by TLC

The components of each fraction 4th, 5th, 6th and 7th(for COS_N, COS_A and COS_B) separated from Sephadex G-25, were analyzed by TLC.TLC techniques can be used to a analyze the end product and identify the mode of action of chitosanases. The reaction products are often determined by visual inspection. The TLC analysis results indicated that each fraction composed of (GlcN), $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$ and $(GlcN)_6$ (Figure 8a&b). The four fractions resulted from Sephadex G-25 contained the same bands, thus Sephadex G-25 could not fractionate the COS. The presence of GlcN indicated that IC_A, IC_B and IC_N were exo and endo type. Little spots were observed between bands. We speculate that these tiny spots correspond to partially acetylated chitooligosaccharides. Cabrera and Cutsem [8] recorded the same result.

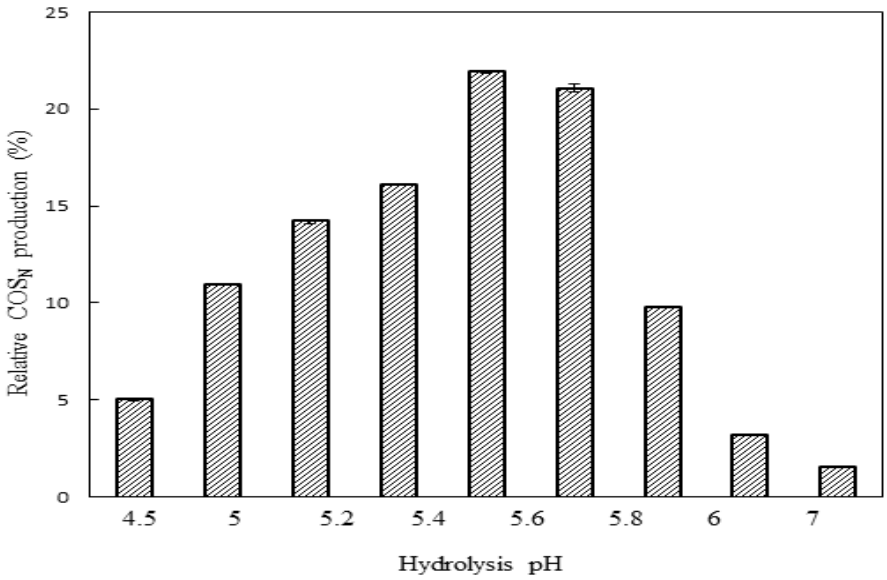

Figure (1): Dependence of chitooligosaccharides production on different pH's. Chitosanase IC_N/chitosan ratio (0.95 U/mg) was incubated at different pH's and 40 °C for 1.5 h in a total volume of 2 ml. Relative COS production was calculated by the chitooligosaccharides (mg) per chitosan (mg) x 100.

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After fractionation of COS_N by ultrafiltration, four kinds of chitooligosaccharides (COS I, COS II, COS III and COS IV) with different molecular size were separated by ultrafiltration by TLC. The TLC analysis results indicated that each fraction was different from the others (Figure 9). Oligosaccharides were moves according to its molecular weight. Little spots were observed between bands. We speculate that these tiny spots correspond to partially acetylated chitooligosaccharides. Cabrera and Cutsem [18] recorded the same result. COS I fraction with molecular weight >100 KDa is considered low molecular weight chitosan (LMC). This was confirmed by TLC for fraction I [22].

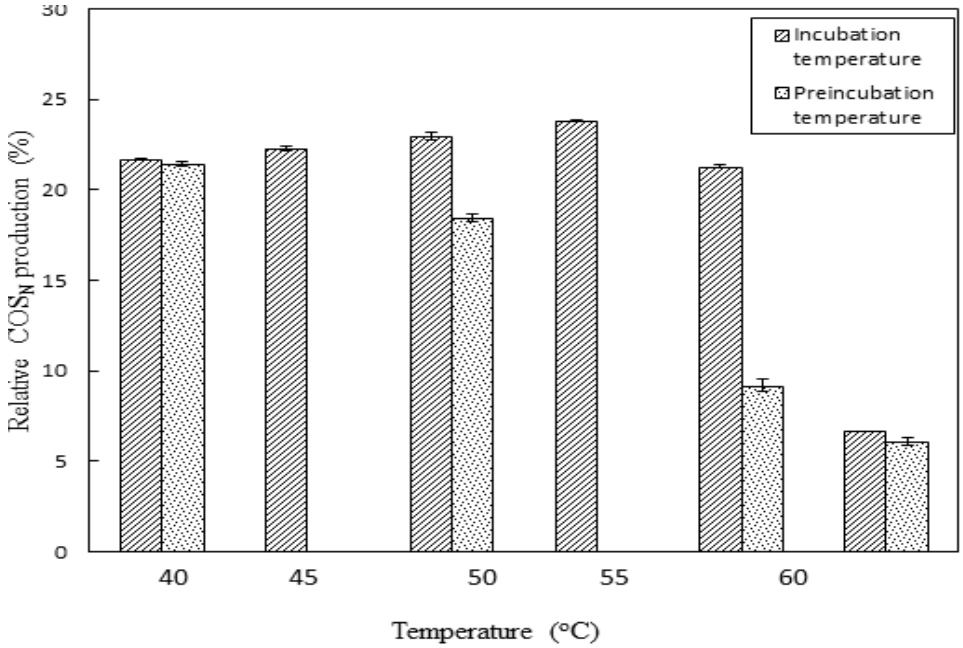

Figure (2): Effect of different temperatures and thermostability on the chitooligosaccharides production. Chitosanase IC_N/chitosan ratio (0.95U/mg) was incubated at various temperatures and pH 5.6 for 1.5 h in a total volume of 2 ml. For thermostability, the enzyme was preincubated at various temperatures for 1 h before enzyme reaction. Relative COS production was calculated by the chitooligosaccharides (mg) per chitosan (mg) x 100.

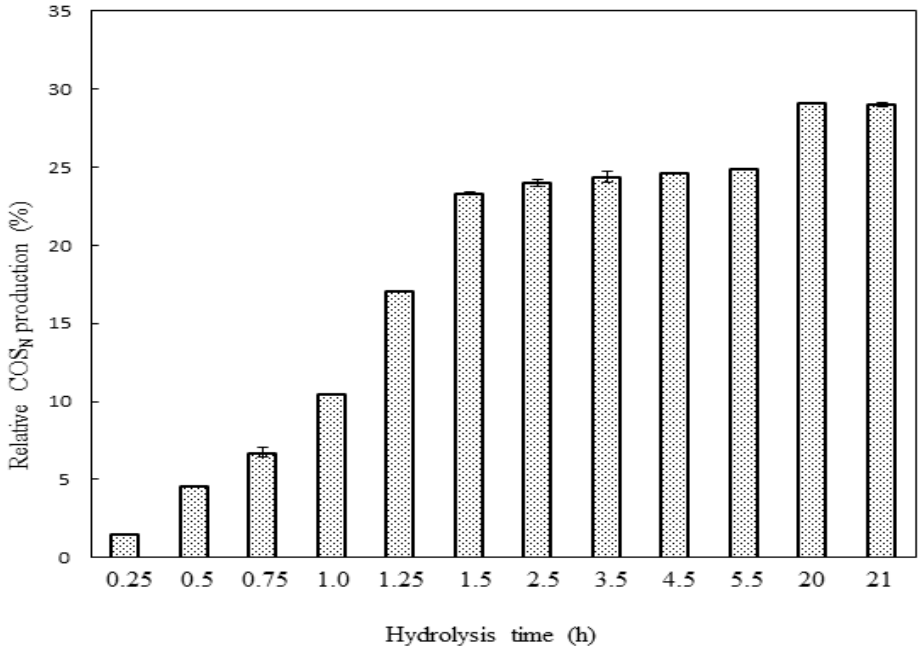

Figure (3): Dependance of Chitooligosaccharides production on different times. Chitosanase ICN/chitosan ratio (0.95 U/mg) was incubated at different times, pH 5.6 and 55 ^{o}C in a total volume of 2 ml. Relative COS production was calculated by the chitooligosaccharides (mg) per chitosan (mg) x 100.

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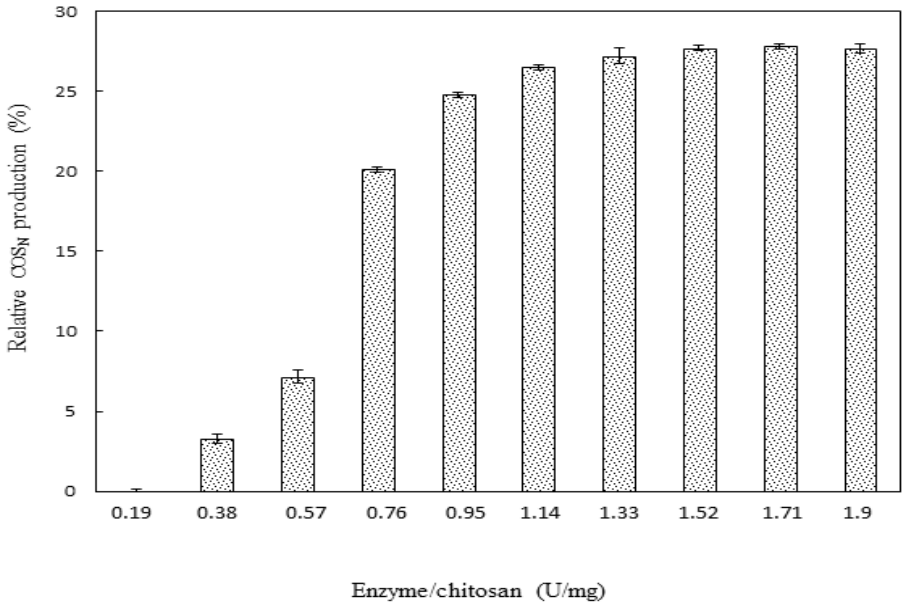

Figure (4): Changes in chitooligosaccharides production at various enzyme/substrate ratios. Various amounts of chitosanase IC_N/chitosan ratio were incubated at 55°C and pH 5.6 for 1.5 h in a total volume 2 ml. Enzyme/substrate ratio was calculated as enzyme activity (U) per chitosan (mg). Relative COS production was calculated by the chitooligosaccharides (mg) per chitosan (mg) x 100.

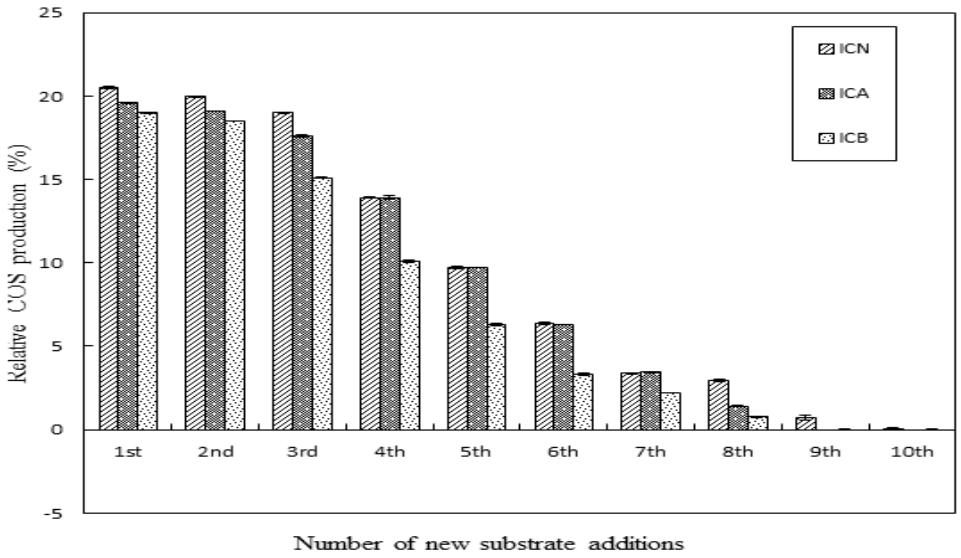

Figure (5): Changes in the chitooligosaccharides (COSs) production following serial addition of new chitosan substrate. Chitosanase/chitosan ratios (0.95U/mg) of IC_N, IC_A and IC_B, respectively, were incubated at pH 5.8 and 40°C in a total volume of 2 ml. Relative COS production was calculated by the chitooligosaccharides (mg) per chitosan (mg) x 100.

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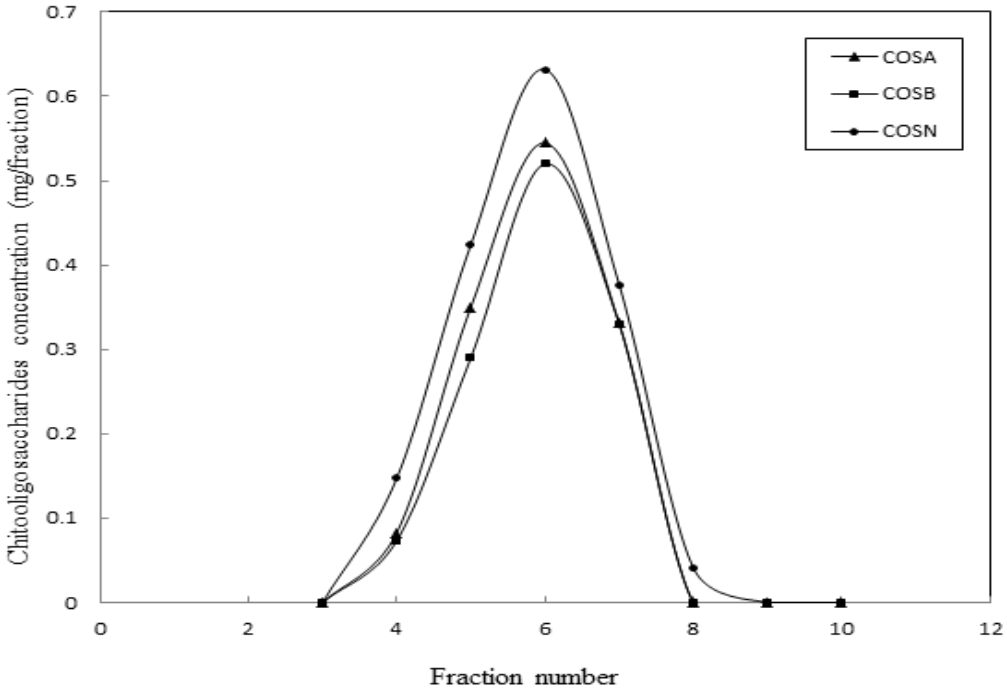

Figure (6): Elution profile of COS_N, COS_A and COS_B on Sephadex G-25.

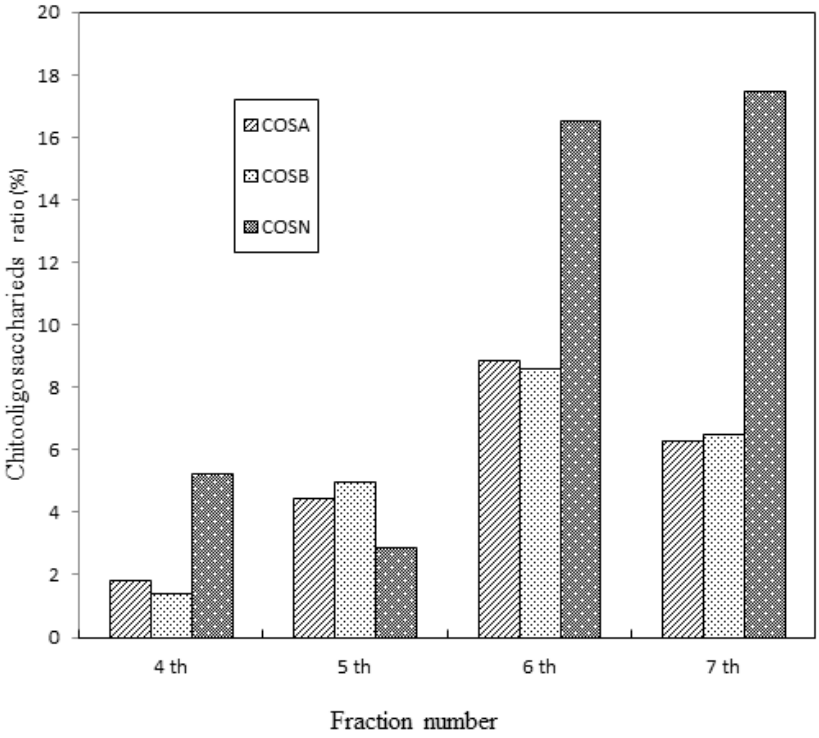

Figure (7): Chitooligosaccharides ratios of COS_N, COS_A and COS_B on Sephadex G-25. COSs ratios were calculated by the COS (mg) per COS (mg dry weight) x 100.

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Table (1): Fractionation of the prepared chitooligosaccharides (2687 mg COS_N) produced from 4800 mg chitosan by immobilized chitosanase IC_N using ultrafilitration technique.

* Relative production was calculated by the chitooligosaccharides (mg) per chitosan (mg) x 100.
* COS ratio (%) was expressed as a percentage of COS (mg) per dry weight (mg) x 100.
* COS yield (%) was expressed as a percentage of the amount of COS in each fraction to total amount of COSs produced.

Fractions	MWCOS (kDa)	COS_N			COS_N Ratio (%)	COS_N Yield (%)
		Conc (mg)	Relative Production (%)	Dry weight (g)		
COS I	>100.0	505	10.5	7.466	6.76	18.8
COS II	100.0-10.0	900	18.75	4.9148	18.31	33.5
COS III	10.0-1.0	840	17.5	1.6892	49.72	31.2
COS IV	<1.0	442	9.2	8.6581	5.1	16.5
Total	_	2687	55.97	22.73	_	100

Figure (8a): TLC analysis of fractions of COS_N from Sephadex G-25. St: glucosamine.

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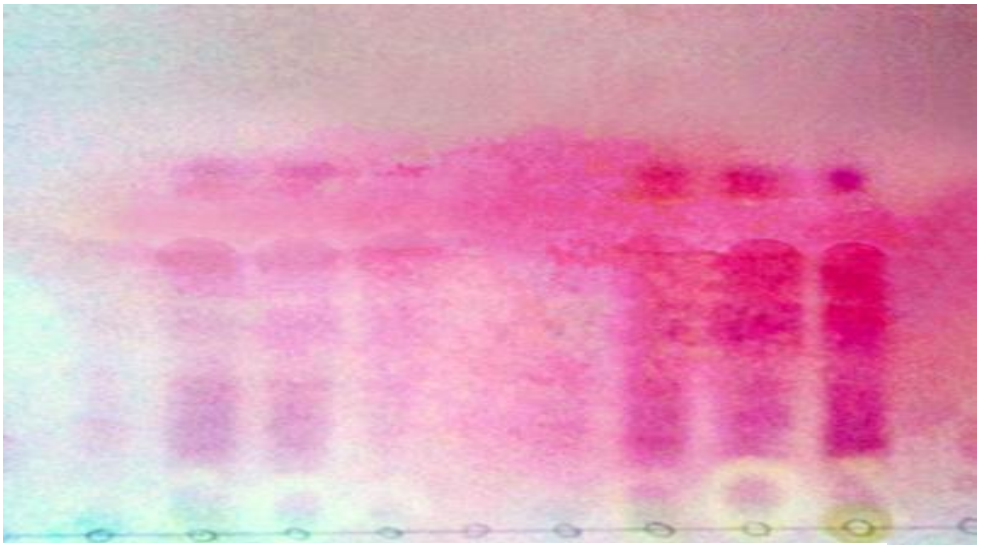
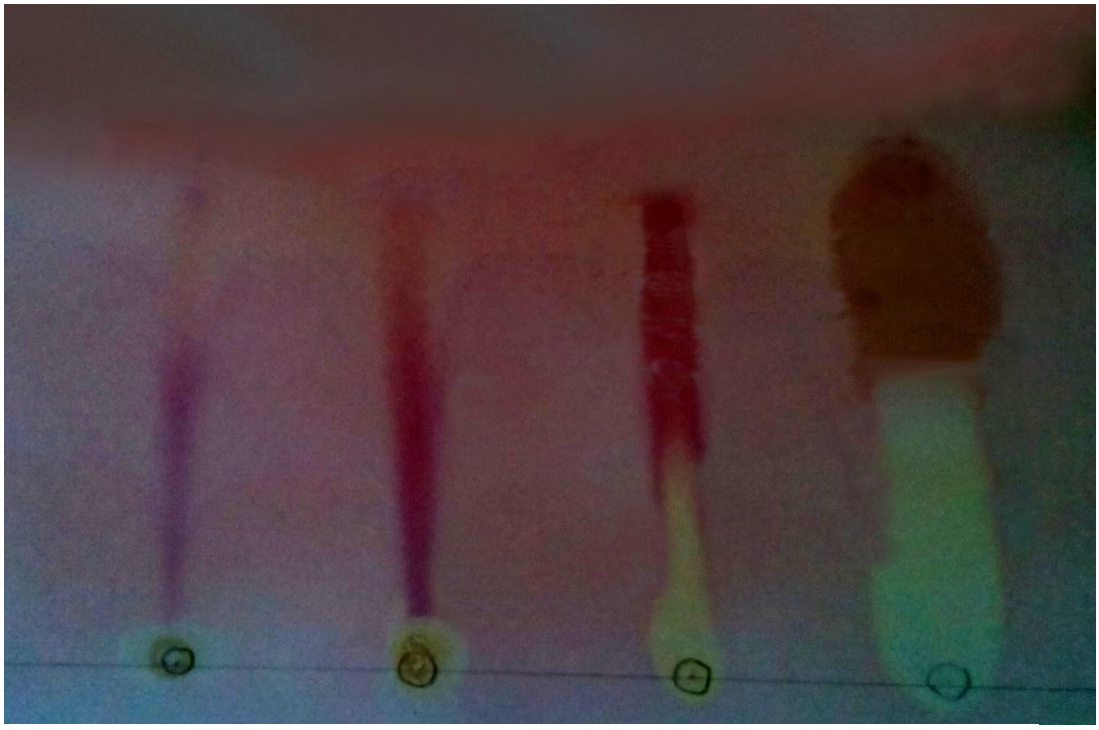


COS_{A4} COS_{A5} COS_{A6} COS_{A7} COS_{B4} COS_{B5} COS_{B6}

Figure (8b): TLC analysis of fractions of COS_A and COS_B from Sephadex G-25.



COSI COSII COSIII COSIV

Figure (9): TLC analysis of fractions of COS_N from ultrafiltration.

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CONCLUSIONS

The present work provides an attractive simple procedure for COS production and separation. Production of COS using immobilized chitosanase at optimum conditions from chitosan was investigated. Optimum conditions for maximal COS production was occurred by using enzyme substrate ratio 0.95 U/mg, at pH 5.6 and 55°C for 1.5 h. To increase the concentration of COS, hydrolysis was carried out using four times addition of chitosan and interesting relative COS production (55.2 %) was obtained. Separation of COS is possible with low cost for large scale yield by ultrafiltration membrane with 100 % recovery. High COS/chitosan ratios of fraction II (18.31) and fraction III (49.72 %) were more than that in fraction I (6.76%) and fraction IV (9.1%). This technique would be useful for an industrial application in medicine.

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