CHAPTER IV
DEACETYLATION OF CHITIN HYDROGEL BY USING SOLUTION PLASMA

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4.1 Abstract

Deacetylation is a chemical reaction used for converting chitin to chitosan. Since native chitin has low reactivity to the deacetylation reaction, chitin hydrogel, an amorphous form of chitin, was applied in this study. Chitin hydrogel was prepared by dissolving native chitin in a calcium chloride-saturated methanol solution and subsequently precipitating in a large amount of water. Solution plasma was introduced to the deacetylation reaction of chitin in order to reduce the NaOH concentration required in the reaction. In the solution plasma system, several highly active species such as hydroxyl radicals, hydroperoxyl radicals, and free electrons were generated. These highly active species might help to facilitate the deacetylation reaction of chitin, which resulted in using a lower concentration of NaOH solution. The deacetylation reaction of chitin hydrogel was carried out by varying concentrations of NaOH in alcohol solutions to 1%, 5%, 10%, and 12%. By using solution plasma, the degree of deacetylation of the chitin hydrogel increased with increasing NaOH concentration as well as the plasma treatment time. The chemical structure and degree of deacetylation of the products were determined by FTIR and NMR. The molecular weight and molecular weight distribution of the obtained

chitosan were investigated by GPC. Moreover, the antimicrobial activity of chitosan obtained from solution plasma method was also evaluated against *E. coli* and *S. aureus*.

Keywords: Chitin hydrogel; Chitosan ; Solution plasma; deacetylation reaction

4.2 Introduction

Solution plasma is a plasma system that generates plasma in liquid. (Takai,2008). This system is able to produce highly active species such as hydroxyl radical, hydroperoxyl radical, free electron, superoxide anion, and atomic oxygen anion. Chitin, poly β-(1-4)-N-acetyl-D-glucosamine, is a polysaccharide consisting of two monomeric units which are N-acetyl-D-glucosamine and D-glucosamine. Chitin which is the second most abundant biomass resource after cellulose was extracted from a by-product of the seafood industry. There are many reports on many applications of chitin such as tissue engineering, wound dressing, drug delivery and cancer diagnosis (Jayakumar *et al.*, 2010, Jayakumar *et al.*, 2011). On the other hand, it has low chemical reactivity due to its high rigid crystalline structure. With the aim of effectively destroying the rigid crystalline structure of chitin and amorphous chitin can be prepared by dissolving chitin in calcium chloride-saturated methanol. (Tamura *et al.*,2007). Furthermore, chitin cannot be dissolved in common solvents. It can be chemically modified to form common solvent-soluble derivatives such as CM-chitin, chitosan, etc. Chitosan is the most studied derivative of chitin because it has been widely used in many applications such as biomedical materials, biodegradable packaging, cosmetics and waste water treatment. However, the problems of chitosan production process are the requirement of high alkali concentration in deacetylation reaction and, as a consequence, the presence of high salt concentration in waste water due to the neutralization of the used alkali by acid. These problems led to a motivation of this study. Plasma has been used in deacetylation reaction of chitin hydrogel to form chitosan in order to avoid the use of high alkali concentration and also to reduce molecular weight of chitosan for better solubility and higher biological activity.

4.3 Experimental

4.3.1 Materials and Chemicals

Shells of *Metapenaeus dobsoni* shrimps were kindly provided by Surapon Foods Public Co., Ltd. (Thailand). 50% (w/w) NaOH solution (Chemical enterprise, commercial grade), glacial acetic acid (CH_3COOH, J.T.Baker, analytical grade), Anhydrous sodium hydroxide (NaOH) pellets, hydrochloric acid (HCl), methanol (CH_3OH), ethanol (CH_3CH_2OH), and propanol ($CH_3CH_2CH_2OH$, Labscan analytical science, analytical grade) were used in this

4.3.2 Preparation of chitosan from shrimp shells

To prepare chitin, shrimp shells were first dried under sunlight for a few days. Then, 1 kg of dried shrimp shells was immersed in 10 L of a 1 N HCl solution with occasional stirring at room temperature for 3 days. The acidic solution was changed daily. The demineralized shrimp shells were subsequently neutralized by deionized water and dried at 60 °C for 48 hr. The demineralized shrimp shells were further deproteinized in a 4 % w/v NaOH solution at a ratio of NaOH solution to shrimp shells of 10:1 with continuous stirring at 80 °C for 4 hr. The obtained chitin was filtrated, neutralized by distilled water, and dried at 60 °C for 24 hr.

4.3.3 Preparation of Chitin hydrogel

There are three steps for preparation of chitin hydrogel. Firstly, to prepare calcium chloride-saturated methanol, 850 g calcium chloride dihydrate was added into 1000 mL of methanol (85%w/v) and refluxed at 60°C for 30 min, followed by standing over night at room temperature and subsequent filtration. Secondly, to prepare chitin solution, 20 g of chitin powder was dissolved in cacluim chloride-saturated methanol by refluxing at 60°C for several hours until the complete dissolution of chitin was accomplished. Finally, to prepare chitin hydrogel, a large amount of distilled water was added into the chitin solution with vigorous stirring to induce the precipitation of chitin hydrogel. Then, the suspension was centrifuged at 12000 rpm for 30 min at 4°C to collect chitin hydrogel. Chitin hydrogel was dialyzed against distilled water for 1 week to remove salt and kept in a refrigerator before use.

4.3.4 Deacetylation of chitin by using solution plasma

The deacetylation reaction of chitin hydrogel was carried out by varying concentrations of NaOH in 90% methanol solutions to be 1%, 5%, 10%, and 12% w/v in order to obtain 2%w/v chitin hydrogel suspension. The chitin hydrogel suspension was added into the plasma reactor and the plasma treatment was operated at the fixed frequency, voltage and pulse width of 12.5 kHz, 2.4 kV and 2 s, respectively. During the plasma treatment, the temperature of chitin hydrogel suspension was at around 50–70°C. The plasma treatment time was one hour for one times of deacetylation reaction. In order to get high value of degree of deacetylation, the deacetylation reaction in association with plasma treatment was performed repeatedly with the change of alkali solutions. The repeated deacetylation reactions with plasma treatment were done up to five times. The deacetylation reaction of chitin hydrogel by using plasma treatment was studied in comparison with the corresponding deacetylation reaction by the conventional heat treatment

4.3.5 Characterization

Chemical structures and degrees of deacetylation of chitin, chitin hydrogel and chitosan were determined by Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR).The degree of deacetylation of chitin and chitosan was calculated by following the method of Sannan *et al.* (1977) and Baxter *et al.* (1999), respectively.

Gel permeation chromatography (GPC) was used to observe any changes in an average molecular weight of plasma treated chitin hydrogel sample at different reaction times and NaOH concentration. The test plasma treated chitin hydrogel sample was filtered through a nylon 66 membrane with the pore size of 0.45 μm (Millipore, USA) before injection into the GPC instrument (Waters, Water 600E) equipped with an refractive index (RI) detector using an ultrahydrogel linear column (molecular weight resolving range of 1.0×10^3 Da to 2.0×10^7 Da). The eluent used in the GPC analysis was an acetate buffer at pH 4.0 (a mixture of 0.5 M CH_3COOH and 0.5 M CH_3COONa). The sample injection volume was 20 μL while the flow rate of the mobile phase was set constant at 0.5 mL min^{-1}. The GPC analysis was done at the chitosan concentration of 2 mg/mL and at the temperature of 30 °C.

Pullulans with the molecular weight in the range of 2.17×10^4 Da to 8.05×10^5 Da were used as standard samples.

The crystalline structure of chitin hydrogel and plasma treated chitin hydrogel was characterized by an X-ray diffractometer (Bruker AXS, D8 advance) operated with the use of Cu Kα as an X-ray source. The WAXD analysis was carried out in a continuous mode with a scan speed of $1° \text{ min}^{-1}$ covering the scanning angle (2θ) from 5° to 50°.

Antibacterial activity of the obtained chitosan from plasma solution treatment was investigated by using the colony forming count method according to the following procedure. A colony of Escherichia coli, a gram negative bacterium, or *Staphylococcus aureus*, a gram positive bacterium, was put into 20 ml. of culture medium containing 0.5%w/v peptone and 0.3%w/v beef extract dissolved in distilled water,. The culture broth was incubated in a shaking incubator at 37 °C and 110 rpm for 24 hr. Then the cell dilution was performed by transferring 0.1 ml. of culture broth to 9.9 ml. of a fresh medium. The dilution process was performed until an appropriate amount of cell concentration was obtained. Next, plasma treated chitin hydrogel was put into a culture broth containg an appropriate amount of bacteria, followed by incubating in a shaking incubator at 37 °C and 110 rpm for 24 hr. After incubation, 0.1 ml. of culture broth was spread on a nutrient agar plate containing 0.3%w/v beef extract, 0.5%w/v peptone and 1.5%w/v agar dissolved in distilled water, followed by incubating at 37 °C for 24 hr. The colony-forming unit (CFU) was calculated and the average was taken of the three plates. The antibacterial activity against either S. aureus or E. coli of the obtained chitosan from plasma solution treatment was determined from the percent reduction in the number of viable bacterial cells

4.4 Results and discussion

4.4.1 Characterization of raw materials

4.4.1.1 Yield of production

In general, chitin from animals occurs associated with other constituents, such as lipids, calcium carbonate, proteins and pigments. So, the shrimp shells were treated with chemicals to extract chitin. The shrimp shells were first cleaned and treated with HCl and NaOH to remove calcium and other minerals, and proteins, respectively. Demineralization occurs according to the following reaction (Belgacem *et al.*, 2008)

$$CaCO_3 + HCl \longrightarrow CO_2 + CaCl_2 + H_2O$$

After dissolving chitin in calcium chloride dihydrate saturated methanol, the product in the form of chitin hydrogel is obtained. The yields chitin and chitin hydrogel production was represented in Table 4.1 and 4.2

Table 4.1 Percent yield of chitin

Samples	Dry weight (%)
Dry shrimp shell	100
Decalcication product	54.17
Deproteination product	20.12
Chitin	25.71

Table 4.2 Percent yield of chitin hydrogel

Samples	Dry weight (%)
Chitin powder	100
Chitin hydrogel	65.8

4.4.1.2 Characterization of chitin and chitin hydrogel

The FT-IR spectra of chitin hydrogel shows the same characteristic peak as chitin, as shown in Figure 4.1, Degree of deacetylation (%DD) of was determined following the method of Sannan *et* al. (1977) which estimated from the ratio of absorbance of amide II band at 1550 cm^{-1} and C-H band at 2878 cm^{1}. In this synthesis, the degree of deacetylation of chitin and chitin hydrogel were calculated to be 34.62% and 35.31%, respectively

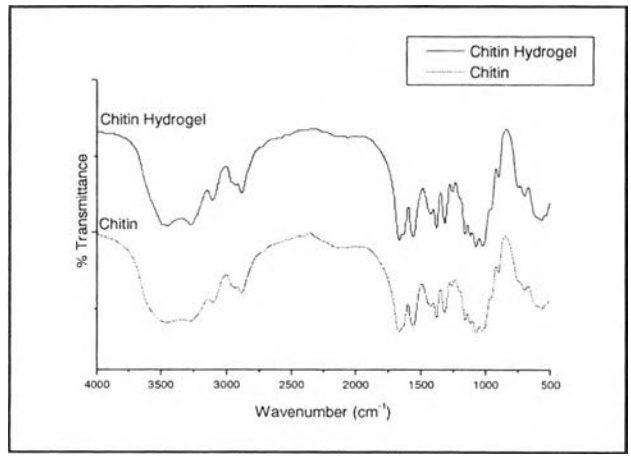


Figure 4.1 FTIR spectra of chitin powder (a) and dried chitin hydrogel (b).

By dissolving chitin powder in calcium chloride-saturated methanol, the rigid crystalline structure of chitin was destroyed and chitin hydrogel was obtained after precipitation of chitin solution in water. Figure4.2 represents XRD spectra of chitin powder and dried chitin hydrogel. This result indicates that dried chitin hydrogel is more amorphous than chitin powder which has sharper peak than dried chitin hydrogel.

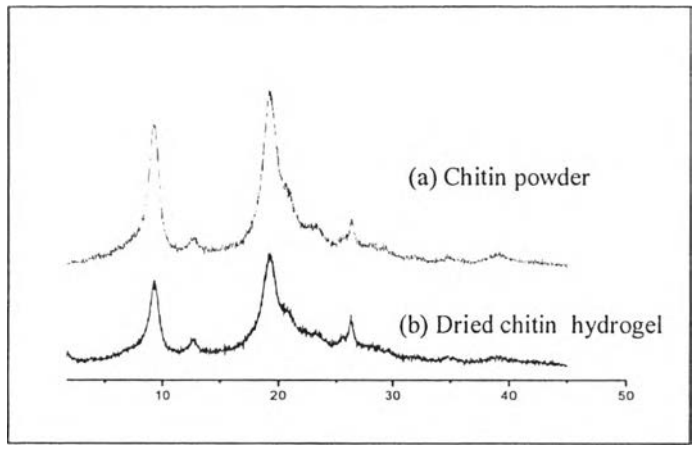


Figure 4.2 XRD spectra of chitin powder (a) and dried chitin hydrogel (b).

It has been proposed that calcium chloride-saturated methanol can dissolve chitin because of the formation of chitin-calcium ion complex, resulting in the disruption of hydrogen bond formation. By dissolving chitin in calcium chloride-saturated methanol solvent system, it has been proposed that calcium ions will form complex with chitin at acetamide group. After adding water into chitin solution, the exchange between water molecule and calcium ions occurs and chitin hydrogel will be obtained. chitin hydrogel formation was shown in Figured 4.3.

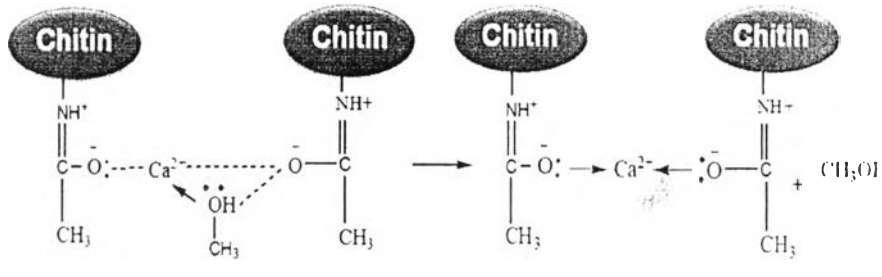


Figure 4.3 Chitin hydrogel formation.

4.4.2 Solvent systems used in deacetylation of chitin hydrogel by using solution plasma system

The solvent systems for deacetylation of chitin hydrogel by using solution plasma were divided into two groups (alcohol solution with and without

NaOH). In case of no NaOH, the ratios of alcohol to water were varied to be 10 to 90, 50 to 50, and 90 to 10. In the presence of NaOH, plasma could be generated only at the ratio of alcohol to water equal to 90 to 10. The NaOH concentrations were increased until plasma could not be genertated. After deacetylation reaction, the values of degrees of deacetylation obtained from plasma treatment were compared to those obtained from the conventional heat treatment at the same condition such as reaction temperature, reaction time. Table 4.3 was found that Regardless of the type of alcohol and the presence or the absence of NaOH, Degree of deacetylation (%DD) of chitin hydrogel were significantly improved in solution plasma system.

Table 4.3 Deacetylation of chitin hydrogel with initial DD=35.13% for 1 hour.

Alcohol	Ratio of Alcohol :H_2O	NaOH Concentration (% w/v)	Reaction Temperature (°C)	Degree of deacetylation	
				Plasma treatment	Conventional heat treatment
Methanol	10:90	0%	72-74	54.17	39.40
	50:50	0%	57-59	45.14	38.33
	90:10	0%	48-49	43.76	36.46
		1%	52-54	55.16	39.90
		5%	55-56	56.89	40.19
		10%	60-62	57.56	40.44
		12%	63-64	59.16	41.73
Ethanol	10:90	0%	76-78	55.50	40.92
	50:50	0%	58-59	47.53	39.11
	90:10	0%	50-52	44.84	36.73
		1%	60-64	56.43	42.98
		5%	70-72	58.84	43.34
Propanol	10:90	0%	81-82	58.64	40.06
	50:50	0%	62-64	48.70	39.11
	90:10	0%	57-59	46.84	36.77

4.4.2.1 Alcohol solution without NaOH system for deacetylation reaction

For alcohol solution without NaOH as a solvent system for deacetylation of chitin hydrogel by using solution plasma. Figure 4.4 represents the operating conditions for plasma treatment in alcohol solution without NaOH

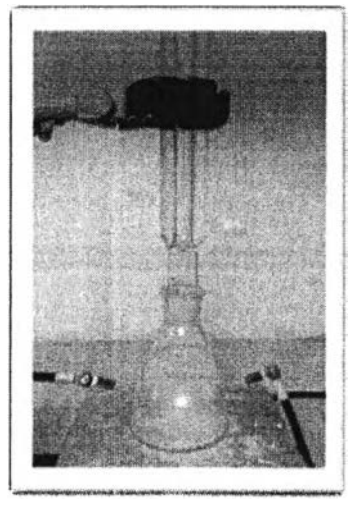


Figure 4.4 The operating conditions for plasma treatment in alcohol solution without NaOH.

These data indicated that the same type of alcohol, using 10% alcohol concentration resulted in the highest value of degree of deacetylation. At the same alcohol concentration, propanol gave the highest and methanol gave the lowest value of degree of deacetylation as shown in Table 4.4.

Table4.4 Effect of type of alcohol on deacetylation of chitin hydrogel with initial degree of deacetylation (%DD) of 35.13% for reaction time of 1 hour

Ratio of Alcohol :H_2O	Alcohol	Reaction Temperature (°C)	Degree of deacetylation	
			Plasma treatment	Conventional heat treatment
10%	Methanol	72-74	54.17	39.40
	Ethanol	76-78	55.50	40.92
	Propanol	81-82	58.64	40.06
50%	Methanol	57-59	45.14	38.33
	Ethanol	58-59	47.53	39.11
	Propanol	62-64	48.70	39.11
90%	Methanol	48-49	43.76	36.46
	Ethanol	50-52	44.84	36.73
	Propanol	57-59	46.84	36.77

Increasing reaction time by using the ratio of alcohol to water equal to 10 to 90 because this ratio shows the highest %DD when treated chitin hydrogel with plasma solution for reaction time of 1 hour. Figure4.5 demonstrates the comparison degree of deactylation of chitin hydrogel between (a) plasma treatment and (b) conventional heat treatment (without NaOH). By using plasma solution in repeated deacetylation reaction, the degree of deacetylation were higher than those of the conventional heat treatment.

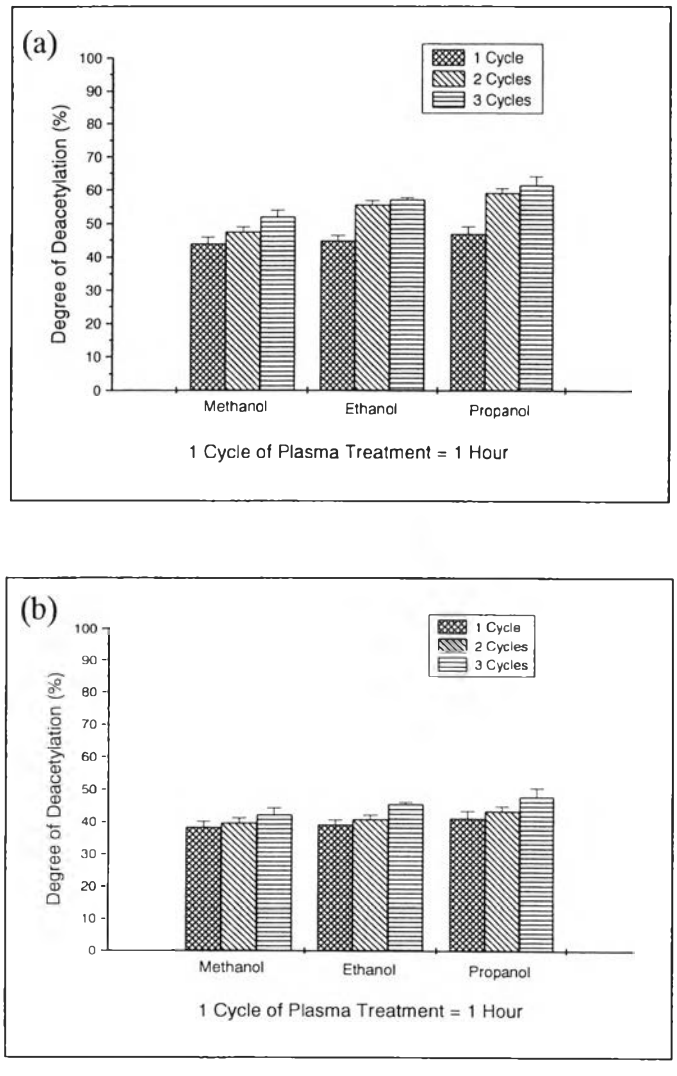


Figure 4.5 Degree of deactylation of chitin hydrogel (a) plasma treatment (without NaOH) (b) conventional heat treatment (without NaOH).

4.4.2.2 Alcohol solution with NaOH system for deacetylation reaction

For the solvent system using alcohol solution with NaOH, methanol/NaOH and ethanol/NaOH solvent systems were evaluated. propanol/NaOH solvent symtem was not studied because NaOH does not dissolve well in propanol. Figure4.6 represents the operating conditions for plasma treatment in alcohol solution with NaOH

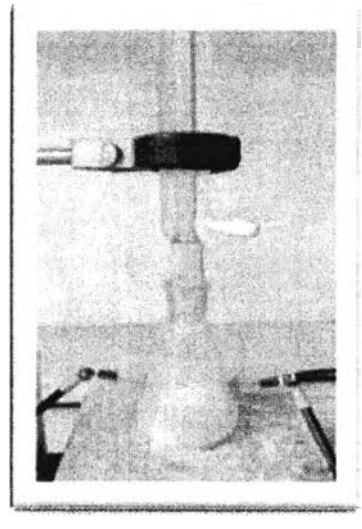


Figure 4.6 The operating conditions for plasma treatment in alcohol solution without NaOH.

When using higher NaOH concentration, the degree of deacetylation can effectively improve under the plasma system. In alcohol/NaOH solvent systems.plasma can occur only at 90% alcohol concentration and NaOH can dissolve in 90% alcohol solution of methanol and ethanol but NaOH cannot dissolve in propanol. Table 4.6 shows the effect of NaOH concentration on deacetylation of chitin hydrogel with initial DD=35.13% for reaction time of 1 hour.

Table 4.5 Effect of NaOH concentration on deacetylation of chitin hydrogel with initial DD=35.13% for reaction time of 1 hour

Alcohol	Ratio of Alcohol :H_2O	NaOH Concentration (% w/v)	Reaction Temperature (°C)	Degree of deacetylation	
				Plasma treatment	Conventional heat treatment
Methanol	90:10	0%	48-49	43.76	36.46
		1%	52-54	55.16	39.90
		5%	55-56	56.89	40.19
		10%	60-62	57.56	40.44
		12%	63-64	59.16	41.73
	10:90	0%	76-78	55.50	40.92
	50:50	0%	58-59	47.53	39.11
Ethanol	90:10	0%	50-52	44.84	36.73
		1%	60-64	56.43	42.98
		5%	70-72	58.84	43.34

The deacetylation reaction of chitin hydrogel was repeated in this NaOH solvent system for 1 to 5 cycle of treatment times. Chitin hydrogel was deacetylated in different concentrations of NaOH (1%,5%,10% and 12% w/v) dissolved in 90% methanol containing $NaBH_4$ (1.0 g/L) to obtain chitosan hydrogel. The degrees of deacetylation (%DD) of the plasma-treated products were determined by FTIR using the equation of Baxter *et al* (1991).

$$\%DD = 100 - [(A1655 / A3450) \times 115] \quad (1)$$

The change in % DD of the plasma-treated products was determined. It was found that % DD of the plasma-treated products increased with the increasing of the number of deacetylation reaction using plasma treatment and the increasing of the NaOH concentration. Chitosan hydrogel prepared by using 12% NaOH concentration for 5 times of deacetylation by plasma treatment had the highest

degree of deacetylation at approximately 78%. Figure4.6. represents the effect of NaOH concentration on degree of deacetylation .

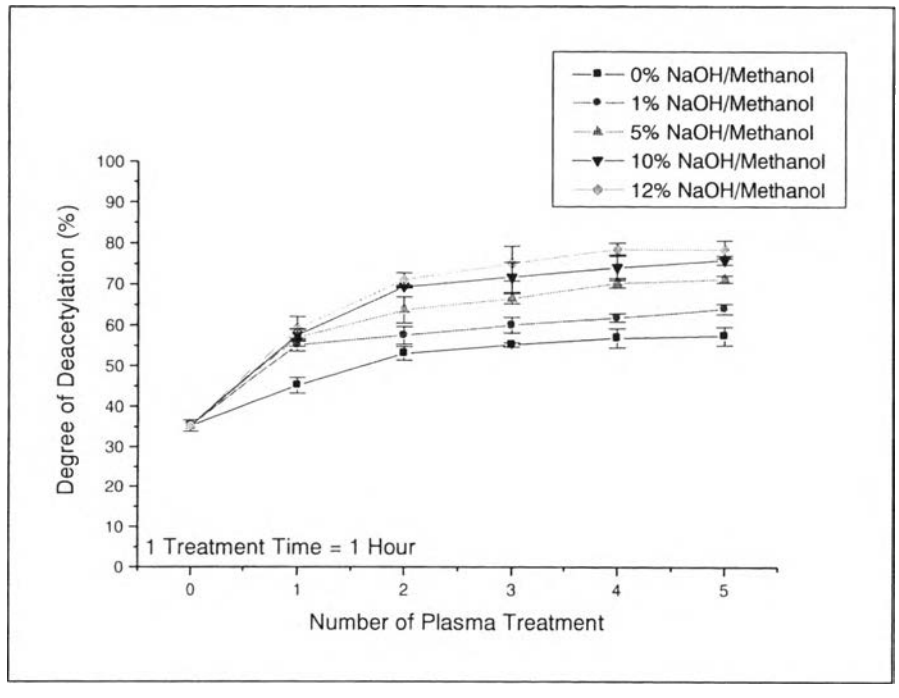
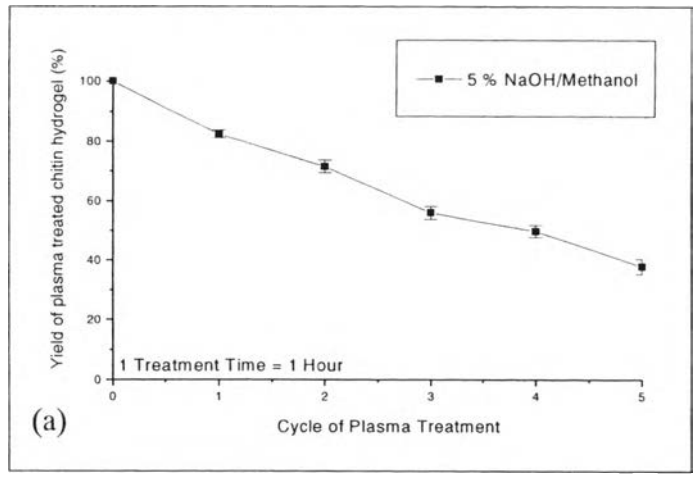
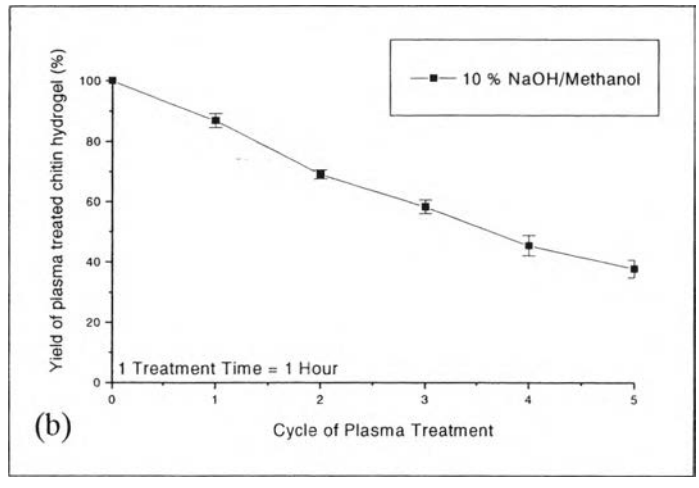


Figure 4.7 The effect of NaOH concentration on degree of deacetylation.

After deacetylation reaction, The yields of plasma-treated chitin hydrogel decreased with the increasing of plasma treatment times and was represented in Figure 4.8





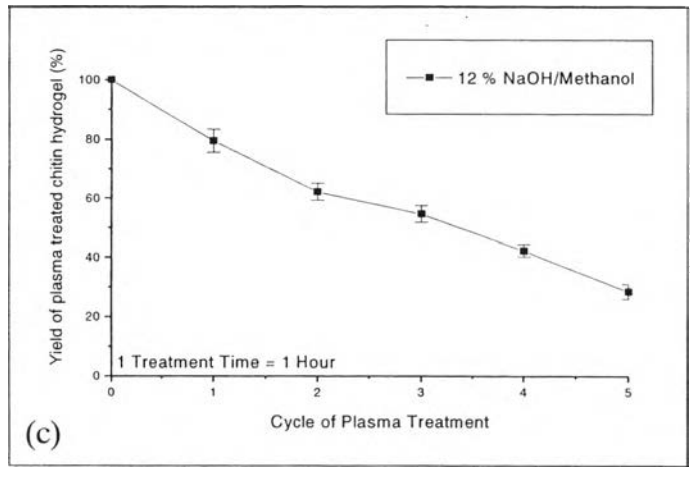
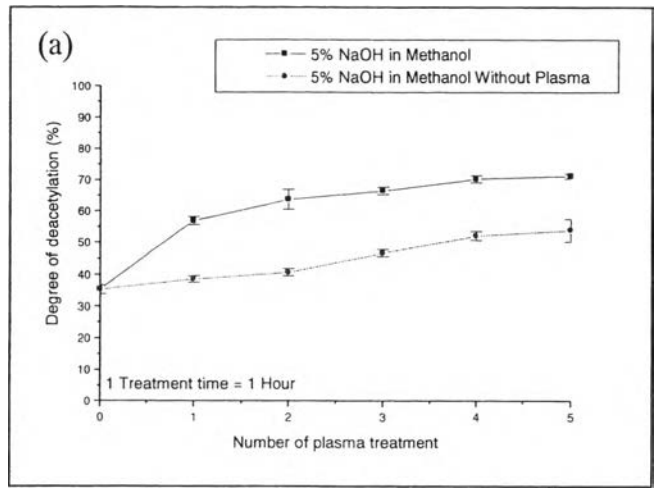
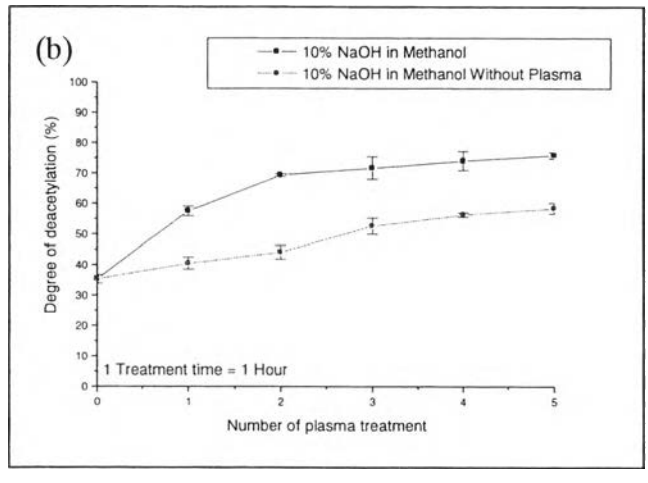


Figure 4.8 The yields of plasma treated chitin hydrogel with (a) 5% NaOH, (b) 10% NaOH, and (c) 12% NaOH.

Moreover, it was found that the deacetylation reaction of chitin hydrogel could proceed more effectively by deacetylation using the solution plasma treatment than by the conventional heat treatment, as shown in Figure 4.9.





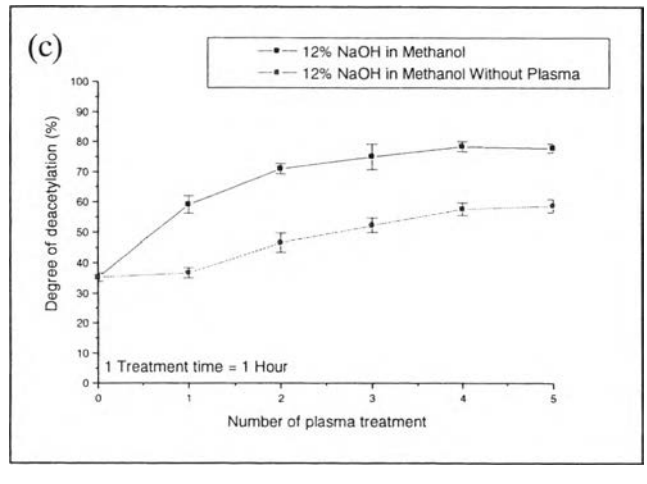
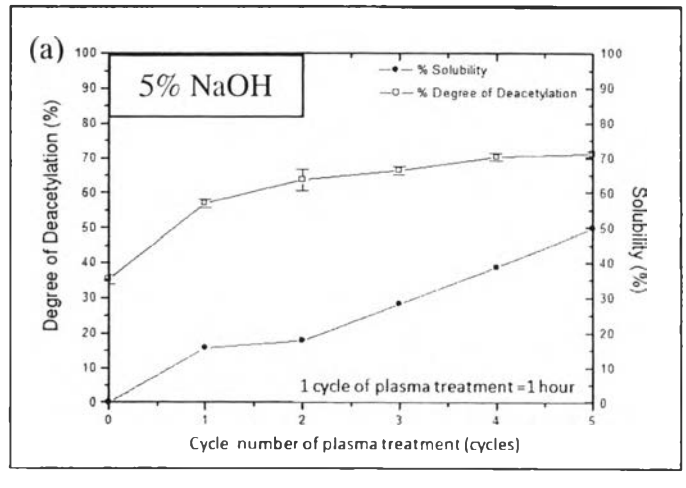
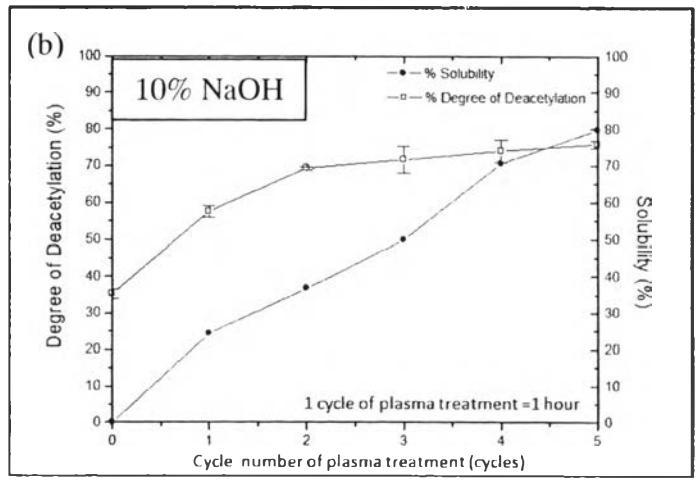


Figure 4.9 Comparison between deacetylation with and without plasma treatment at different NaOH concentrations (a) 5% NaOH, (b) 10% NaOH, and (c) 12% NaOH.

In this study, It has been proposed that chitin hydrogel can be converted to chitosan due to chitosan is soluble in aqueous organic acids, e.g. acetic. Figure 4.10 shows the solubility of plasma-treated chitin hydrogel in 2% acetic acid with (a)5% NaOH ,(b)10% NaOH,(c)12% NaOH concentration. The solubility of chitosan obtained by deacetylation in association with plasma treatment using 12% NaOH concentrations was determined as a function of the number of deacetylation by plasma treatment. The results indicated that the solubility of plasma-treated chitin hydrogel increased with the increasing of the number of deacetylation by plasma treatment. Chitin hydrogel deacetylated with 12% NaOH for 4 and 5 times of deacetylation by plasma treatment was completely soluble in 2% acetic acid solution.





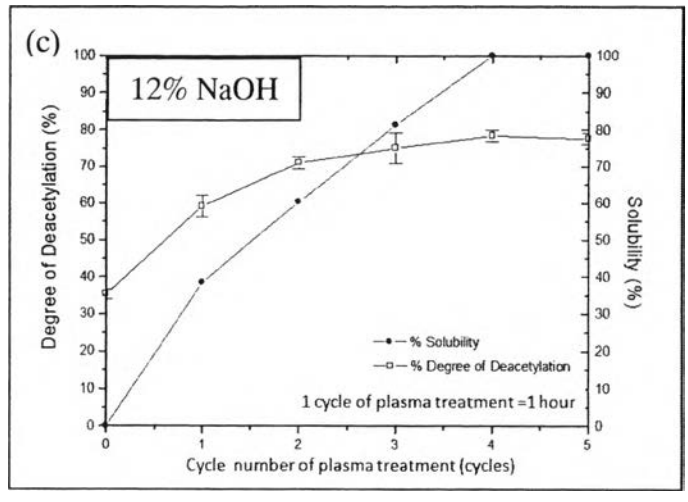


Figure 4.10 Solubility of plasma-treated chitin hydrogel in 2% acetic acid with (a)5%NaOH ,(b)10% NaOH,(c)12% NaOH concentration.

4.5 Characterization of the obtained chitosan product from plasma solution treatment

4.5.1 Fourier transformed infrared spectroscopy (FTIR)

The FTIR spectroscopy was used to investigate any changes in the chemical structure of chitosan sample after the deacetylation process, as shown in Figure 4.11. Chemical structure of chitin hydrogel and plasma-treated chitin hydrogel deacetylation by using different NaOH concentrations in 90% methanol solution were investigated by FT-IR. From the FT-IR spectra, the transmittance band at the wavenumber of 1655 cm^{-1}, which is the characteristic peak of carbonyl in acetyl group, decreased with the increasing of NaOH concentration.

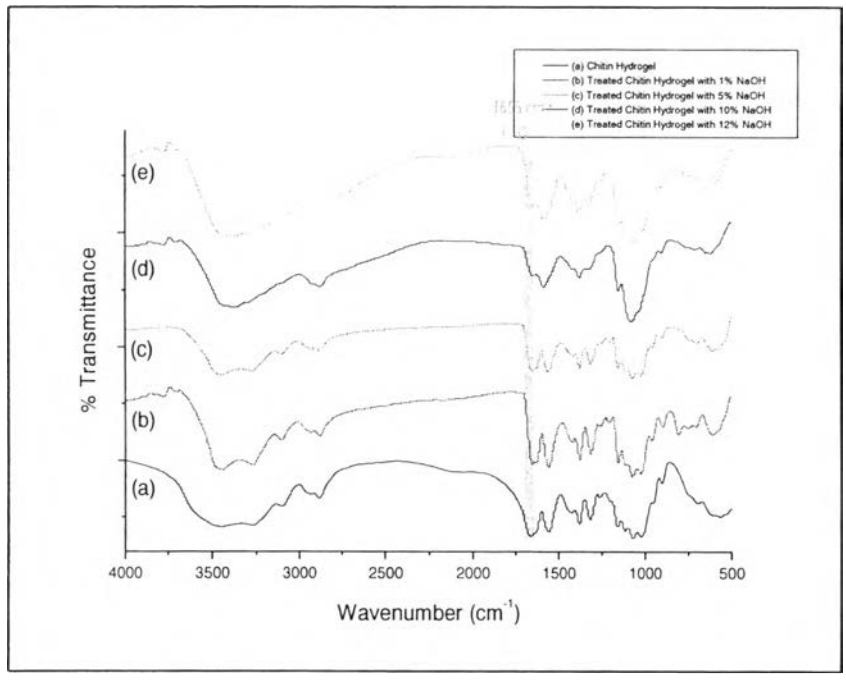


Figure 4.11 FT-IR spectra of chitin hydrogel (a), treated chitin hydrogel in deacetylation reaction with plasma treatment by using different NaOH concentration at 1% (b), 5%(c), 10%(d) and 12%(e) w/v in methanol solution.

4.5.2 Nuclear magnetic resonance (NMR)

Chemical structures of the plasma treated chitin hydrogel was confirmed H^1 NMR technique. Figure 4.12 represents the NMR spectra of plasma treated chitin hydrogel by deacetylation reaction with12% NaOH in 90% MeOH solution for 5 hr.

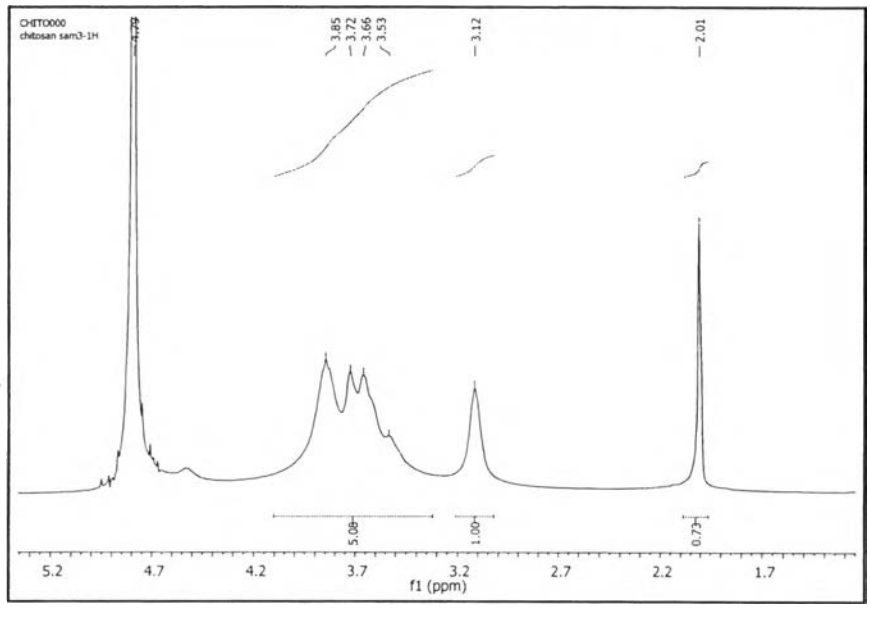


Figure 4.12 NMR spectra of plasma treated chitin hydrogel by deacetylation reaction with12% NaOH in 90% MeOH solution for 5 hr.

The degree of deacetylation of plasma treated chitin hydrogel was calculated by H-NMR following the method of Lavertu *et al.* (2003).

$$\%DD = 1 - \frac{\text{Integral area } CH_3}{3\times \text{Integral area } H_2} \times 100 \qquad (2)$$

In this study, the degree of deacetylation of the obtained chitosan product from plasma solution treatment were determined by using FTIR and NMR technique. The result shows that the value of degree of deacetylation obtained form NMR was nearly the value of degree of deacetylation obtained from FTIR. Comparison of determination of degree of deacetylation by using FTIR and NMR

technique for deacetylation of chitin hydrogel by using solution plasma for 5 hours at 12% NaOH in 90% MeOH solution is shown in Table 4.6

Table 4.6 Comparison of determination of degree of deacetylation by using FTIR and NMR technique

Characterization	Degree of deacetylation	Reference
FTIR	78.46 %	Baxter *et al.*,1992
NMR	77.88 %	Lavertu *et al.*,2003

Deacetylation reaction of chitin to form chitosan occurs by the nucleophilic attach of hydroxide anion at the carbonyl carbon at the acetamide group of chitin. The bond between carbonyl carbon and nitrogen at acetamide group is broken with the removal of acetate anion while amino group is formed instead of acetamide group. The mechanism of deacetylation reaction is shown in Figure 4.13.

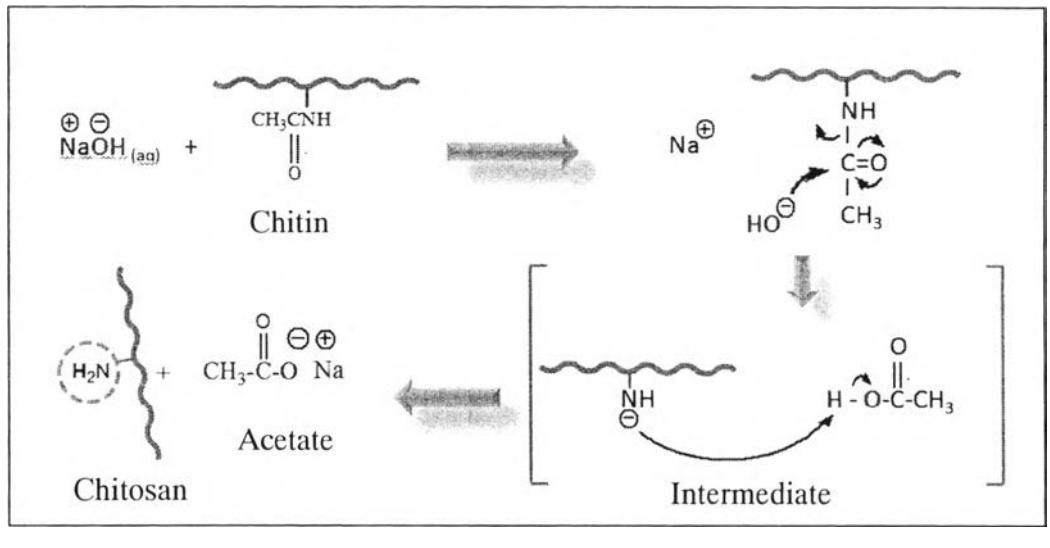


Figure 4.13 Mechanism of deacetylation reaction.

4.5.3 Gel permeation chromatography (GPC)

Molecular weight of plasma-treated chitin hydrogel was determined by gel permeation chromatography (GPC). Table 4.7 represents that the molecular weights of plasma-treated chitin hydrogel decreased from 245,880, 230,840 to 220,149 at the plasma treatment of 3, 4, and 5 hours, respectively. Table 4.8 demonstates that the molecular weights of plasma-treated chitin hydrogel slightly decreased with the increasing of NaOH concentrations.

Table 4.7 The molecular weight of treated chitin hydrogel in 12% NaOH concentration with different times by using solution plasma

Condition	Treated chitin hydrogel 3 hours	Treated chitin hydrogel 4 hours	Treated chitin hydrogel 5 hours
M_n	91,141	88,942	87,689
M_w	245,880	230,840	220,149
PDI	2.69781	2.59541	2.51059

Table 4.8 The molecular weight of plasma-treated chitin hydrogel obtained by varying NaOH concentrations at 5 hours of plasma treatment time

Condition	Plasma-treated chitin hydrogel 5% NaOH	Plasma-treated chitin hydrogel 10% NaOH	Plasma-treated chitin hydrogel 12% NaOH
M_n	103,134	105,596	87,689
M_w	228,674	222,543	220,149
PDI	2.21726	2.10749	2.51059

In addition to deacetylation reaction, the main chain scission at glycosidic bond also occurred due to alkaline depolymerization. For deacetylation by plasma treatment under alkali condition, various radical reactive species generated by plasma might involve in glycosidic bond cleavage. Figure 4.14 shows the degradation of chitin hydrogel under plasma treatment. of alkaline depolymerization (a) and the reaction with reactive species generated by solution plasma (b).

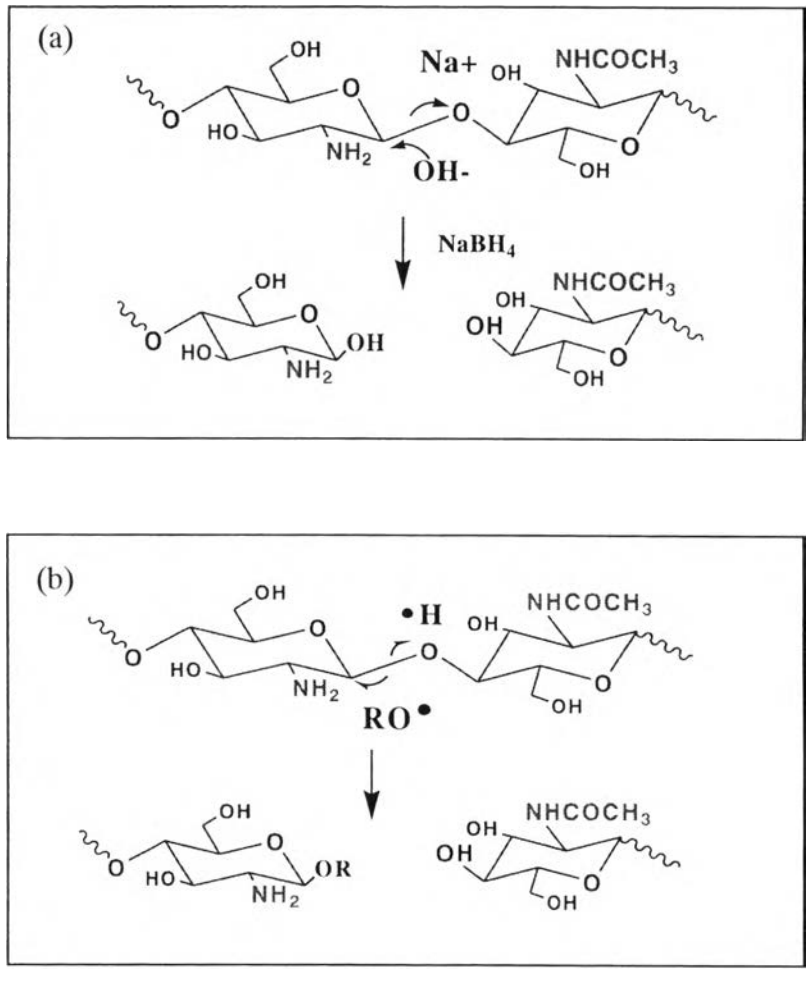


Figure 4.14 Degradation of chitin hydrogel under plasma treatment of alkaline depolymerization (a) and the reaction with reactive species generated by solution plasma (b).

4.5.4 X-ray diffraction experiment (XRD)

XRD spectra of plasma-treated chitin hydrogel after repeated deacetylation for 5 times in 12% NaOH/methanol solution and chitin hydrogel is shown in Figure 4.15. The result shows that XRD spectras show two peaks approximately at 10 and 2θ, but the peaks of treated chitin hydrogel were higher and sharper than chitin hydrogel, indicating a higher level of crystallinity .

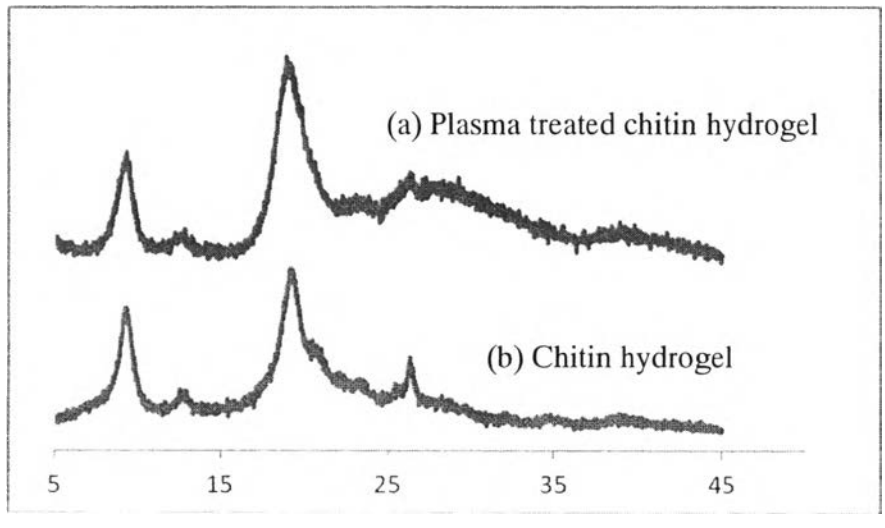


Figure 4.15 XRD spectra of plasma-treated chitin hydrogel after repeated deacetylation for 5 times in 12% NaOH/MeOH solution (a) and chitin hydrogel (b).

4.5.5 Antibacterial activity

Figure 4.16 and Figure 4.17 indicated that plasma-treated chitin hydrogel deacetylated repeatedly for 4 and 5 times with %DD of 78 had antibacterial activity by inhibiting the growth of E. coli and S. aureus due to the effective protonated amino group of the obtained chitosan. The percents of bacterial reduction rate of E. coli and S. aureus are indicated in Table 4.9 and Table 4.10 respectively.

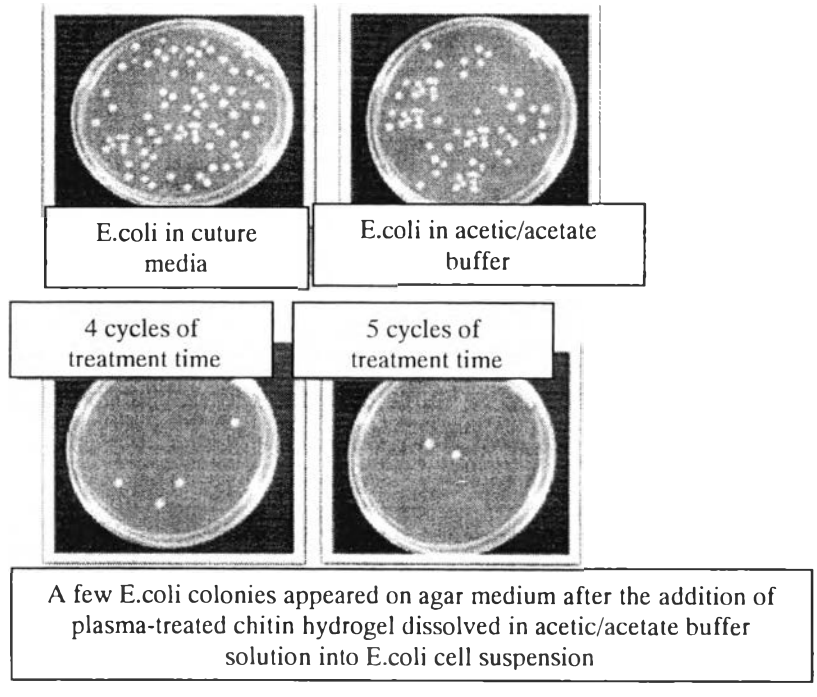


Figure 4.16 Antibacterial activity of the obtained chitosan from plasma solution treatment against E.coli

Table 4.9 Antibacterial activity of the obtained chitosan from plasma solution treatment against E.coli

Cycles of treatment time.	Bacterial Reduction Rate (%)
4	90.9 ± 6.4
5	96.96 ± 7.3

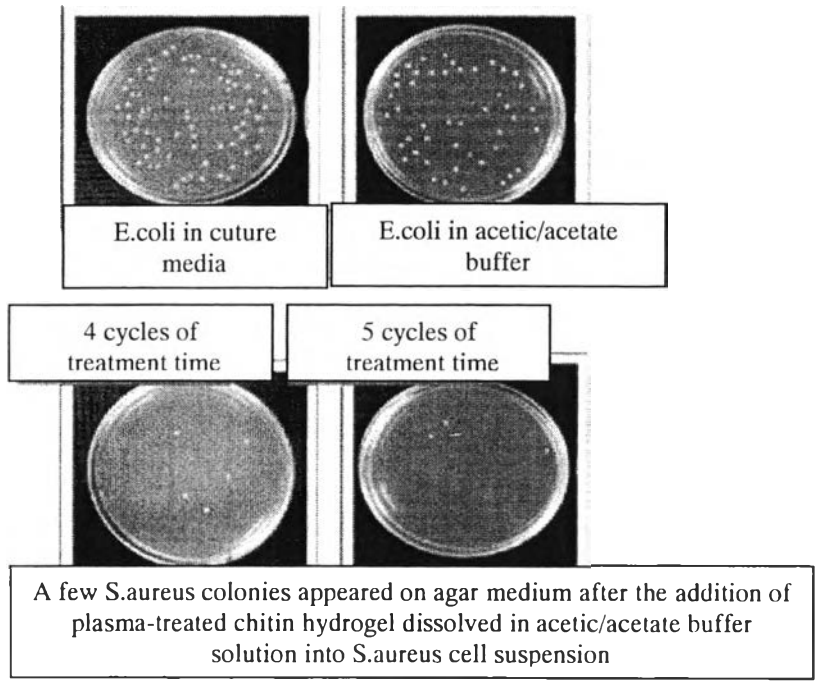


Figure 4.17 Antibacterial activity of the obtained chitosan from plasma solution treatment against S.aureus

Table 4.10 Antibacterial activity of the *obtained chitosan* from plasma solution treatment against S.aureus

Cycles of treatment time.	Bacterial Reduction Rate (%)
4	89.79 ± 5.4
5	95.91 ± 5.1

4.6 Comparison between chitosan production obtained from solution plasma treatment and obtained from literature review and both have the same degree of deacetylation

4.6.1 Reducing high salt concentration in chitosan process

In general, the high values of degree of deacetylation can be achieved only at high temperature and using high concentrations of alkali solutions. These data are shown in Table 4.11.

Table 4.11 Chitosan production obtained from literature review

Alkaline/solvent	Temperature (°C)	Degree of Deacetylation	Reference
40% NaOH aqueous solution	80	70 - 99 %	Kurita *et al.*,2001
40% KOH in ethanol and monoethyleneglycol	80	75 - 83 %	Broussignac *et al.* 1968
40% NaOH aqueous solution (*Ultrasonic radiation*)	60 – 100	40 – 95%	Zhang *et al.*,2007

These concentrated alkali solution generate high salt concentration in waste water, resulting in the high cost of waste water treatment and the high production cost of chitosan.

Problem of chitosan production process is the presence of high salt concentration in waste water due to the neutralization of the used alkali by acid. In this study, plasma has been used in deacetylation reaction of chitin hydrogel to form chitosan in order to reduce the use of high alkali concentration .

4.6.2 Degree of deacetylation

The degrees of deacetylation (%DD) of the plasma-treated chitin hydrogel with 12% NaOH concentration and original chitosan with 50% NaOH were determined by FTIR using the equation of Baxter *et al* (1991). The results show in Table 4.12.

Table 4.12 Degree of deacetylation of plasma treated chitin hydrogel and original chitosan

Type of chitosan	Degree of deacetylation (%DD)				
	1	2	3	Average	SD
Plasma treated chitin hydrogel 12% NaOH (solution plasma)	76.08	78.97	80.32	78.46	2.17
Original chitosan 50% NaOH	77.61	77.62	78.34	77.86	0.42

4.6.3 Molecular weight

After deacetylation reaction, The molecular weight of plasma-treated chitin hydrogel and original chitosan decreased with increasing of plasma treatment times however molecular weight of plasma treated chitin hydrogel with 12% NaOH was lower than molecular weight of original chitosan with 50% NaOH. The molecular weight of plasma-treated chitin hydrogel and original chitosan were determined by gel permeation chromatography (GPC). These data represented in Table 4.13

Table 4.13 The molecular weight of treated chitin hydrogel in 12% NaOH concentration with different times by using solution plasma and original chitosan with 50% NaOH concentration

Condition	Treated chitin hydrogel 3 hours	Treated chitin hydrogel 4 hours	Treated chitin hydrogel 5 hours	Original chitosan with 50% NaOH
	with 12% NaOH			
M_n	91,141	88,942	87,689	256,740
M_w	245,880	230,840	220,149	498,080
PDI	2.69781	2.59541	2.51059	1.90106

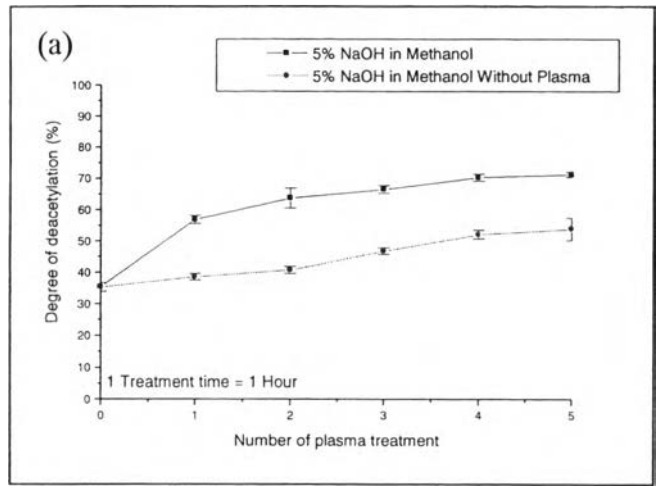
4.7 Comparison between chitosan production obtained from solution plasma treatment and obtained from conventional heat treatment by using the same condition

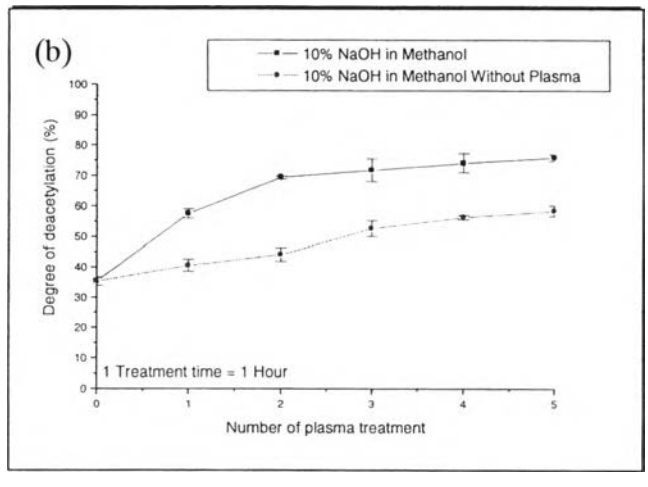
It has been proposed that the deacetylation reaction of chitin hydrogel could proceed more effectively by deacetylation using the solution plasma treatment than by the conventional heat treatment. Table 4.14 shows the comparison between chitosan production obtained from solution plasma treatment and obtained from conventional heat treatment by using the same condition

Table 4.14 Comparison between chitosan production obtained from solution plasma treatment and obtained from conventional heat treatment by using the same condition

Plasma solution treatment	Conventional heat treatment
Lower NaOH concentration	Higher NaOH concentration
low salt concentration in waste water	high salt concentration in waste water
Lower temperation of reaction	Higher temperation of reaction
At low temperature, this system can generate OH^- from NaOH , alcohol and water	At low temperature, this system can generate OH^- from NaOH only

Figure 4.18 demonstrates the comparison of degree of deacetylation of chitin hydrogel between using solution plasma treatment and conventional heat treatment with NaOH concentration and Figure 4.19 for without NaOH concentration.





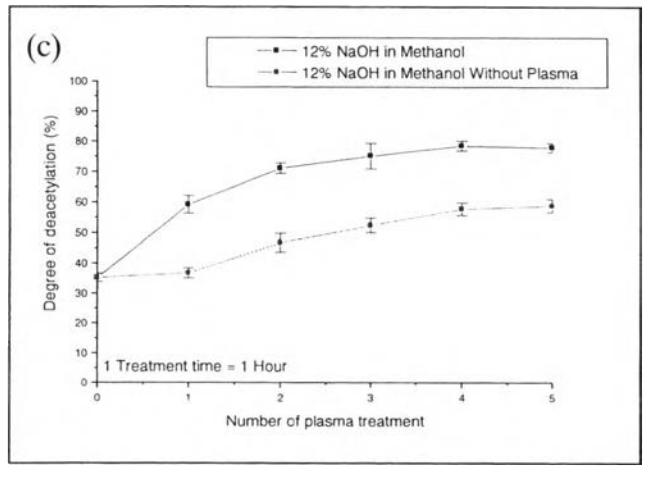


Figure 4.18 Comparison between deacetylation with and without plasma treatment at different NaOH concentrations (a) 5% NaOH, (b) 10% NaOH, and (c) 12% NaOH.

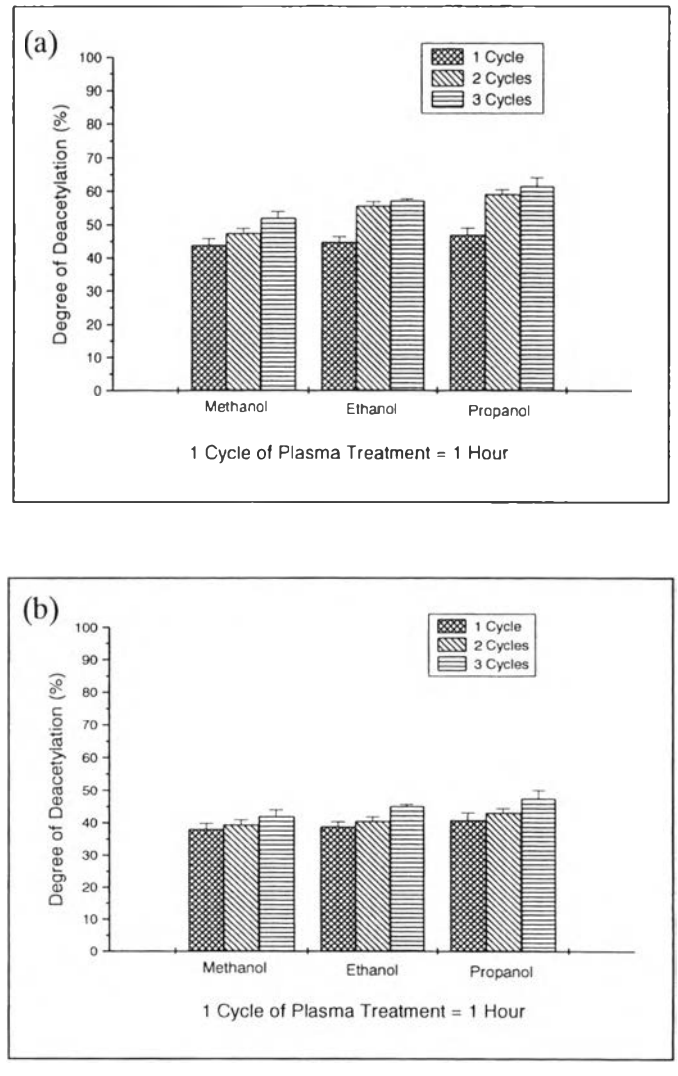


Figure 4.19 Degree of deactylation of chitin hydrogel (a) plasma treatment (without NaOH) (b) conventional heat treatment (without NaOH).

The degree of deacetylation of chitosan production obtained from solution plasma treatment was higher than chitosan production obtained from conventional heat treatment because the solution plasma technique is able to produce highly active species. At low temperature, this system can generate OH^- from NaOH , alcohol and water, as indicated in Figure 4.20 , Figure 4.21 , Figure 4.22 respectively. But at low temperature, the conventional heat treatment can generate OH^- from NaOH only.

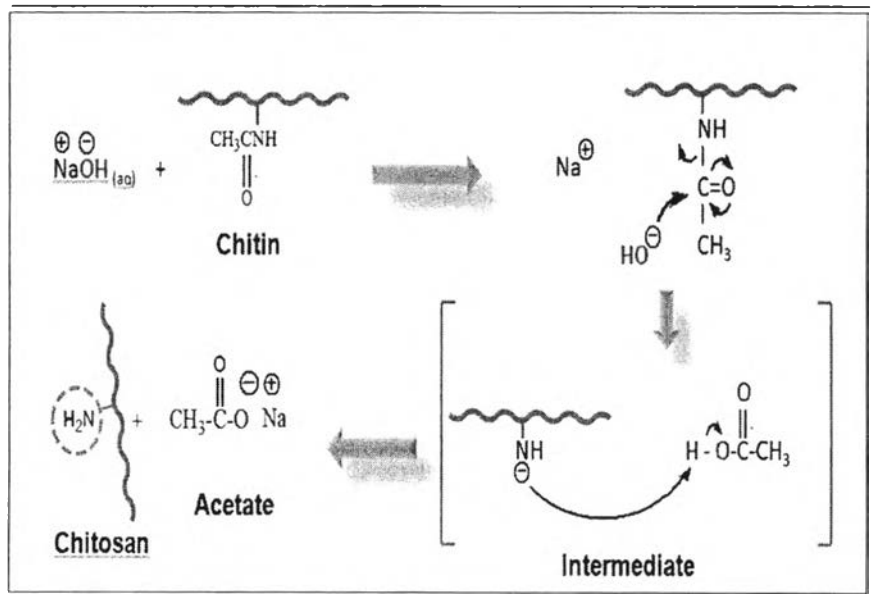


Figure 4.20 Mechanism of deacetylation reaction of chitin hydrogel by using solution plasma treatment in NaOH concentration.

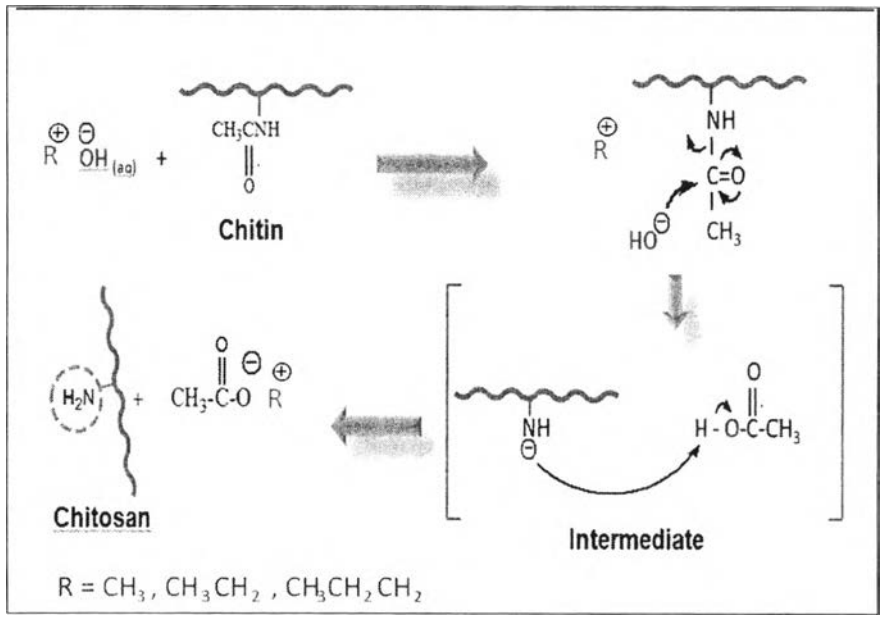


Figure 4.21 Mechanism of deacetylation reaction of chitin hydrogel by using solution plasma treatment in alcohol solvent.

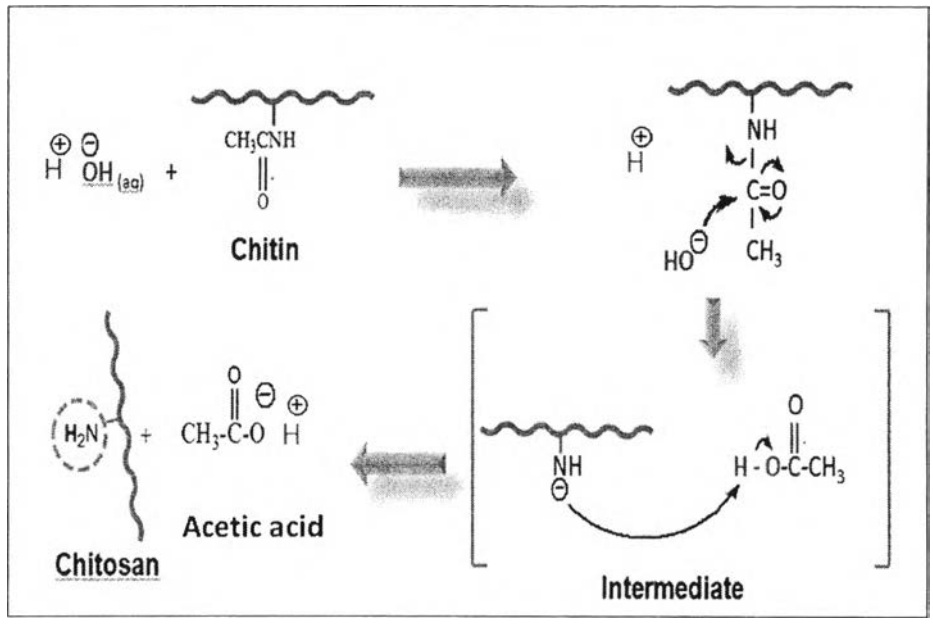


Figure 4.22 Mechanism of deacetylation reaction of chitin hydrogel by using solution plasma treatment in water.

4.8 Conclusion

For deacetylation in association with plasma treatment, the conversion of chitin to chitosan could be achieved with diluted NaOH solution. The factors that have an influence on the degree of deacetylation are type of alcohol, ratio of alcohol to water, NaOH concentration, and the number of deacetylation. By usinndg solution plasma to produce chitosan from chitin hydrogel, not only *N*-deacetylation reaction was promoted, degradation caused by the residual NaOH and various radical reactive species generated by solution plasma also occurred resulting in the reduction of molecular weight of chitosan that facilitated the solubility and enhanced the antibacterial activity of chitosan.

4.9 Acknowledgement

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Advisor: Assoc. Prof. Ratana Rujiravanit

Dr. Chutima Vanichvattanadecha

4.10 References

Cho Y-W, Jang J, Park CR, Ko S-W. (2000) Preparation and solubility in acid and water of partially deacetylated chitins. Biomacromolecules,1:609–14.

Goosen, M.F.A. (1997) Applications of chitin, chitosan, Pennsylvania: Technomic Publishing.

Helbert, W., Cavaille, J. Y., and Dufresne, A. (1996) Polymer Composition, 17, 4, 604.

Jayakumar, R., Prabaharan, M., Nair, S.V., Tokura, S., Tamura, H., and Selvamurugan, N. (2010) Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. Progress in Materials Science, 55, 675–709.

Jayakumar, R., Prabaharan, M., Nair, S.V., Tokura, S., Tamura, H., and Selvamurugan, N.(2010) Biomedical applications of chitin and chitosan based nanomaterial-A short review. Carbohydrate Polymer, 82, 227-232.

Jayakumar , R., Prabaharan, M., Nair, S.V., Tokura, S., Tamura, H., and Selvamurugan, N. (2011) Biomaterials based on chitin and chitosan in wound dressing applications. Biotechnology Advances, 29, 322-337.

Khor, E., and Lim, L.Y. (2003) Implantable applications of chitin and chitosan. Biomaterials, 24, 2339–2349.

Kurita K. Chemistry and application of chitin and chitosan.(1995) Polymer Degradation and Stability,59,117–20.

Kurita K (1997) Chitin and chitosan derivatives. In: Arshady R (ed) Desk reference of functional polymers: syntheses and applications. Washington,DC American Chemical Society, pp 239–259

Kurita K (2006) Introduction of biologically active branches through controlled modification reactions of chitin and chitosan. In: Uragami T, Tokura S (eds) Material science of chitin and chitosan. Kodansha Scientific, Tokyo

Kumar, M.N.V.R. (2000) A review of chitin and chitosan applications. Reactive & Functional Polymers, 46, 1–27.

Li, J., Revol, J.F., and Marchessault, R. H. J. (1996) Applications of chitin and chitosan-derivatives for the detoxification of water and wastewater. Colloid Interface Science, 183,
365.

Lu, Y., Weng, L., and Zhang, L. (2004) Morphology and Properties of Soy Protein Isolate Thermoplastics Reinforced with Chitin Whiskers. Biomacromolecules, 5, 1046-1051.

Marchessault, R. H., Morehead, F. F., and Walter, N. M. (1959) Liquid Crystal Systems from Fibrillar Polysaccharides. Nature, 184, 632.

Marumatsu, K., Masuda, S., yoshihara, Y., and Fujisawa, A. (2003) In vitro degradation behavior of freeze-dried carboxymethyl-chitin sponges processed by vacuum-heating and gamma irradiation. Polymer Degradation and Stability, 81, 327–332

Morin, A. and Dufresne, A. (2002) Nanocomposites of Chitin Whiskers from Riftia Tubes and Poly(caprolactone). Macromolecules, 35, 2190–2199.

Nair, K.G., and Dufresne, A. (2003) Crab shell chitin whisker reinforced natural rubber nanocomposites 1. Processing and swelling behavior. Biomacromolecules, 4, 657-665.

Paillet, M. and Dufresne, A. (2001) Chitin Whisker Reinforced Thermoplastic Nanocomposites. Macromolecules, 34, 6527–6530.

Pillai, C.K.S., Paul, W., Sharma, C.P. (2009) Chitin and chitosan polymers: Chemistry, solubility and fiber formation. Progress in Polymer Science, 34, 641–678.

Raabe, D., Sachs, C., and Romano, P. (2005) The crustacean exoskeleton as an example of a structurally and mechanically graded biological nanocomposite material. Acta Materialia, 53, 4281–92.

Revol, J.F., and Marchessault, R.H. (1993) In vitro chiral nematic ordering of chitin crystallites. International Journal Biological Macromolecules, 15, 329–35.

Rinaudo, M. (2006) Chitin and chitosan: Properties and applications. Progress in Polymer Science, 31, 603–632.

Sandford, PA., Sjak-Braek, G., and Anthonsen, T.(1989) Chitin and chitosan. London: Applied Science, 51–69.

Sriupayo, J., Supaphol, P., Blackwell, J., and Rujiravanit, R. (2005) Preparation and characterization of α-chitin whisker-reinforecd chitosan nanocomposite films with or without heat treatment. Carbohydrate Polymers, 62:130-136.

Takai, O. (2008). Solution plasma processing (SPP). Pure and Applied Chemistry, 80, 2003–2011.

Tamura H, Nagahama H, Tokura S.(2006) Preparation of chitin hydrogel under mild conditions. Cellulose,13:357–64.

Tamura H, Tsurutaa Y, Itoyamab K,WorakitkanchanakulW, Rujiravanit R, Tokura S. (2004) Preparation of chitosan filament applying new coagulation system. Carbohydrate Polymers,56,205–11.

Tamura H, Nagahama H, Tokura S. (2007) Preparation of chitin hydrogel under mild conditions. Japan Apply Polymer Science,104,3909–16.

Tamura H, Sawada M, Nagahama H, Higuchi T, Tokura S. (2006) Influence of amide content on the crystal structure of chitin. Holzforschung;60,480–4.

Tokura S, Miura Y, Johmen M, Nishi N, and Nishimura S.I. (1994) Journal Control Release, 28, 235–41.

Tokura, S., Baba, S., Uraki, Y., Miura, Y., Nishi, N., and Hasegawa, O. (1990) Controlled functionalization of the polysaccharide chitin. Carbohydrate Polymers, 13, 273–81.

Uragami T, Kurita K, Fukamizo T (eds) (2001) Chitin and chitosan in life science. :Tokyo, Kodansha Scientific, pp 178–185

Vasnev, V.A., Tarasov, A.I., Markova, G.D., Vinogradova, S.V., and Garkusha, O.G. (2006) Synthesis and properties of acylated chitin and chitosan derivatives. Carbohydrate Polymers, 64, 184–189

Watanabe, K., Saiki, I., Uraki, Y., Tokura, S., and Azuma, I. (1990) Regioselective Conjugation of Chitosan with a Laminin-related Peptide. Chemical Pharmaceutical Bull, 38, 506–9.

Wongpanit, P., Sanchavanakit, N., Pavasant, P., Supaphol, P., Tokura, S., and Rujiravanit, R. (2005). Preparation and Characterization of Microwave-treated Carboxymethyl Chitin and Carboxymethyl Chitosan Films for Potential Use in Wound Care Application. Macromolecular Bioscience, 5, 1001–1012

Yi H, Wu L-Q, Bentley WE, Ghodssi R, Rubloff GW, Culver JN, et al. Biofabrication with chitosan. Biomacromolecules 2005;6:2881–94.

Zaini, M. J., Fuad, M. Y. A., Ismail, H., Mansor, M. S., and Mustafah, J. (1996) Preparation and properties of polypropylene composites reinforced with wheat and flax straw fibres: Part II Analysis of composite microstructure and mechanical properties. Polymer International, 40, 51-55.