IJPSR (2018), Volume 9, Issue 1 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 21 April, 2017; received in revised form, 11 August, 2017; accepted, 17 September, 2017; published 01 January, 2018

HEMICELLULASE - MEDIATED HYDROLYSIS OF CHITOSAN: AN EFFICIENT METHOD TO SYNTHESIZE WATER SOLUBLE CHITOSAN

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Keywords:

Low molecular weight chitosan, Water soluble chitosan, Enzymatic hydrolysis, Improved crystallinity, Hemicellulase, Aqueous solubility

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ABSTRACT: Chitosan is a natural biopolymer that has been extensively used traditionally for the treatment of various ailments and as an efficient vehicle or carrier for fabrication, encapsulation and target-specific delivery of a wide range of pharmacological moieties for improved therapeutic efficacy. Despite of numerous characteristic features, the pharmaceutical significance of chitosan is limited due to its poor water solubility. The present study was aimed to investigate the impact of enzyme (hemicellulase)-mediated hydrolysis on the physicochemical characteristics including solubility, physicochemical nature, crystallinity, contact angle/wettability and surface morphology of the WSC. The sustainability of enzymatic method was evaluated by testing aqueous solubility and carrying out fourier-transform infrared spectroscopy (FTIR), x-ray diffraction (XRD), thermogravimetric analysis (TGA), contact angle measurement and scanning electron microscopic (SEM) analysis. Results of solubility studies revealed that hemicellulase-mediated hydrolysis significantly enhanced the aqueous solubility and wettability of the chitosan and exhibited smaller contact angle (θ) in comparison to the unmodified LMWCS. FTIR analysis showed that WSC synthesised via enzymatic hydrolysis exhibited ultra-low molecular weight and increased deacetylation degree in comparison to the unmodified LMWCS. Moreover, there was no change in chemical nature of all the functional groups. XRD analysis indicated that the increased in crystallinity of the WSC after enzymatic degradation, however, solubility of WSC was not affected. These findings indicated that hemicellulase-mediated hydrolysis of LMWCS is an efficient alternative method to synthesise WSC without altering its physicochemical nature and pharmaceutical integrity.

INTRODUCTION: Chitosan [α-(1→4)-2-amino-2-deoxy-β-D-glucan] is a natural biopolymer that has received great attention as a pharmaceutical excipient and as a carrier, vehicle, and ligand in drug delivery research and for the development of efficient controlled release delivery systems [1 - 3].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(1).80-87

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(1).80-87

Moreover, its intrinsic properties such as biodegradability, biocompatibility, special adhering feature to the mucosal surface, penetration enhancing by opening epithelial tight-junctions and immune stimulating activity has increased attention to exploit chitosan as protein antigens delivery system [4, 5]. Degradation of chitosan has also shown to be nontoxic, non-immunogenic and non-carcinogenic which makes it as a promising candidate for the development of a variety of novel formulations and drug delivery systems [6, 7]. The advantages of chitosan is such that, they can be easily processed into different forms such as

membranes, sponges, gels, scaffolds, microparticles, nanoparticles and nanofibers for a variety of biomedical applications such as drug delivery, gene therapy, tissue engineering and wound healing. As a key component of many cosmetic, pharmaceutical, and nutraceutical products, chitosan has been numerously used to synthesise a wide range of advanced health materials and delivery systems [8] including nanoparticles [9, 10] polymeric films [11], nanogel [12], nanospheres [13], nanofibers [14], nanocomposites, porous membranes [15] and mucoadhesive sheets.

Despite of several characteristic features, the pharmaceutical significance of chitosan is limited due to its poor water solubility. The native chitosan dissolves only in aqueous acetic acid solution that induces undesirable cytotoxicity in the encapsulated formulations and increases sensitivity towards bioactive macromolecules such as peptide or protein drugs, genetic material and anticancer drugs [16, 17]. Many chitosan derivatives are water soluble in a wide pH range and have unique biological activities and physicochemical properties [18]. In addition, Tao *et al.* [19] reported that use of water soluble chitosan (WSC) reduces mean particle size of the microspheres and has a preventive effect on diet-induced obesity.

Chitosan is insoluble in either water or most of organic solvent, although it is soluble in aqueous diluted acids; the poor solubility of chitosan becomes the major limiting factor in its utilization, such as the application of chitosan in biology, in which many enzyme assays are performed at neutral pH. If WSC could be prepared in a simple manner, it is expected that the biological and physiological potentials of chitosan would be developed dramatically.

Degree of deacetylation is one of the important characteristics which will influence the performance of chitosan in its applications. The degree of deacetylation determines the amount of free amino groups present in the polysaccharides [20]. Chitosan is a semi-crystalline polymer possesses a degree of crystallinity which reflects the degree of deacetylation [21]. On the other hand, molecular weight of chitosan determines various physicochemical characteristics of chitosan such as solubility, viscosity, adsorption on solids, elasticity and tear strength [22]. In addition, the film-forming properties and crystal size of chitosan are also dependent on its molecular weight.

Generally, the crystallinity of chitosan film/membrane is increased with a decrease in its molecular weight. The increase in crystallinity will also enhance the aqueous solubility of chitosan and thus overall applicability in designing and formulating drug delivery systems.

There are two most commonly employed methods to synthesize low molecular weight chitosan (LMWCS) which include 1) acidic degradation and 2) enzymatic degradation [23]. Acidic degradation involve acidic hydrolysis of the chitosan by using hydrochloric acid (HCl), nitric acid (HNO_3), and phosphorous acid (H_3PO_4) into water soluble oligomers with low polymerization degree [24]. Chemical modification through acidic hydrolysis is not generally preferred as it may change the fundamental skeleton of chitosan, thus, may lost its intrinsic physicochemical and biochemical properties [25].

In this study, we used enzymatic degradation method to synthesise WSC having higher degree of deacetylation and aqueous solubility. The suitability and sustainability of enzymatic method was investigated by testing the synthesized WSC for various physicochemical characterizations including aqueous solubility, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), thermogravimetric analysis (TGA), contact angle measurement, and scanning electron microscopic (SEM) analysis.

MATERIALS AND METHODS:

Chemicals and Reagents: Low molecular weight chitosan from the source of crab shells and hemicellulase enzyme (Cat. No. H2125) from *Aspergillu sniger* with activity 1.5 unit/mg were purchased from Aldrich, USA. Glacial acetic acid and sodium hydroxide (NaOH) were purchased from R and M Chemicals, U.K. Hydrochloric acid (HCl) was purchased from Lab-Scan, Thailand. Deuterium oxide, D_2O (99.9 atom %) and deuterated acetic acid (Acetic-d3 acid-d), CD_3COOD (99.5 atom %) were purchased from Sigma Aldrich, USA.

D-glucosamine hydrochloride and *N*-acetyl-glucosamine were obtained from Fluka Biochemical, Switzerland. All other chemical, reagents and solvents used were of analytical reagent grade.

Hemicellulase - Mediated Synthesis of WSC: In this method, WSC was synthesised from LMWCS by using hemicellulase enzyme using a previous method 19 with minor modifications. Briefly, 1 g of LMWCS was dissolved in 50 mL of 2% v/v acetic acid. The mixture was then stirred for 3 h, and the pH was adjusted to 4.5 using 1M NaOH or HCl. The mixture was then maintained at a temperature of 40 ºC by placingin a water bath for 6 h. After that, hemicellulase (20% w/v) solution was added to the reaction mixture to trigger enzymatic hydrolysis reaction. The reaction mixture was then removed from the water bath and the pH was adjusted to 5.5 using 1M NaOH in order to neutralize the acidic contents. The reaction mixture was then boiled for about 10 min to denature the enzyme followed by the concentration of the mixture to 1/5 using a rotatory evaporator at a temperature of 60 °C. Afterward, the remaining mixture was freeze-dried to obtain WSC powder.

Characterisation of Synthesised WSC: The synthesised WSC was characterized for aqueous solubility, FTIR, XRD, TGA, contact angle measurement, and SEM analysis.

Aqueous Solubility: In this experiment, the aqueous solubility of the synthesised WSC was evaluated in comparison with LMWCS. Briefly, 1 g of WSC was dissolved into 100 mL of distilled water at room temperature (25 ± 3 °C). The solution was then stirred for a period of 10 - 15 min and was visually examined. A similar procedure was used to prepare aqueous solution of LMWCS.

FTIR Analysis: FTIR spectra were scanned using IRAffinity-1S Fourier transform infrared (FTIR) spectrophotometer (SHIMADZU, Japan). Briefly, a small quantity (2 - 3 mg) of WSC was mixed with 200 - 300 mg potassium bromide (KBr) and was compressed to form transparent pellets using a hydraulic press. Notably prior to form transparent pellets, the materials were dried in an oven, overnight. Similar procedure was used to prepare transparent pellets of LMWCS. Finally, the resulting pellets were scanned in transmission mode in a spectral region of 4000 - 500 cm^{-1} using a resolution of 1 cm^{-1} and 32 co-added scans. The FTIR data were analysed by using Origin 6.1 software to locate characteristic peaks.

XRD Analysis: The physical characteristics of WSC and LMWCS were also evaluated through XRD analysis. Prior to scanning, the powder samples were heated up to 300 °C with a heating rate of 10 °C/min. XRD spectra was obtained by using Bruck D8 Advance powder diffractometer with Bragg-Brentano (θ, 2θ) geometry with CuKα radiation and a function of a function of the 2θ range of 20° - 80° at 40 kV and 30 mA.

TGA Analysis: TGA measurements were carried out using Netzsch TG 209 F1 Phoenix®. Briefly, 5 mg of freeze-dried powdered sample of WSC was placed in a covered aluminum pan and was heated from 30 - 700 ^{0}C with a heating rate of 10 °C/min under a dynamic nitrogen atmosphere at a flow rate of 25 mL/min. A similar procedure was used to scan the lyophilized dried powder of LMWCS.

Contact Angle Measurement: Contact angles of WSC and LMWCS samples were measured by "Sessile Drop" method using static contact angle instrument (OCA 15 EC, Data physics, Germany). Prior to perform the experiment, the powdered samples were compressed into disc forms using a hydraulic press. A drop of liquid (pure distilled water) was dispensed onto the sample surface and the contact angle was calculated. Calculation of the contact angle took place by mathematical expression which was applied in the droplet shape in order to obtain the tangent value of the interface. The procedure was performed at 25 ± 2 °C and a relative humidity of 55 ± 5%.

SEM Analysis: SEM analysis was conducted using Quanta FEG 450 SEM. Briefly, a small amount of WSC powder was placed on a carbon coated double cello tape. This tape was then placed on a stub before being coated with platinum. The image was then taken under 200× and 500 × magnifications. A similar procedure was used for LMWCS.

Statistical Analysis: All the resulting data was statistically analysed using paired t-test or student t-test.

The data is presented as mean ± standard deviation (S.D). Ap value of less than <0.5 was considered as significant.

RESULTS AND DISCUSSION:

Characterization of Synthesized WSC:

Solubility Study: This study was performed with the aim to enhance aqueous solubility of chitosan. hemicellulase-hydrolysed chitosan (WSC) showed a transparent, clear, sticky viscous solution after 15 min of stirring compared to a cloudy, opaque solution of LMWCS when dissolved in water (**Fig.1)**. These results indicated the efficiency of enzyme-hydrolysis method to produce water-soluble oligomers of chitosan.

A1 **A2** **B1** **B2**

FIG. 1: SOLUBILITY STUDY OF WATER SOLUBLE CHITOSAN (WSC) (B1, B2) COMPARED TO LOW MOLECULAR WEIGHT CHITOSAN (LMWCS) (A1, A2). A CLEAR SOLUTION OF WSC WAS OBSERVED IN COMPARISON WITH OPAQUE, CLOUDY SOLUTION OF LMWCS

FTIR Analysis: FTIR spectra of the synthesised WSC and LMWCS are shown in **Fig. 2**.

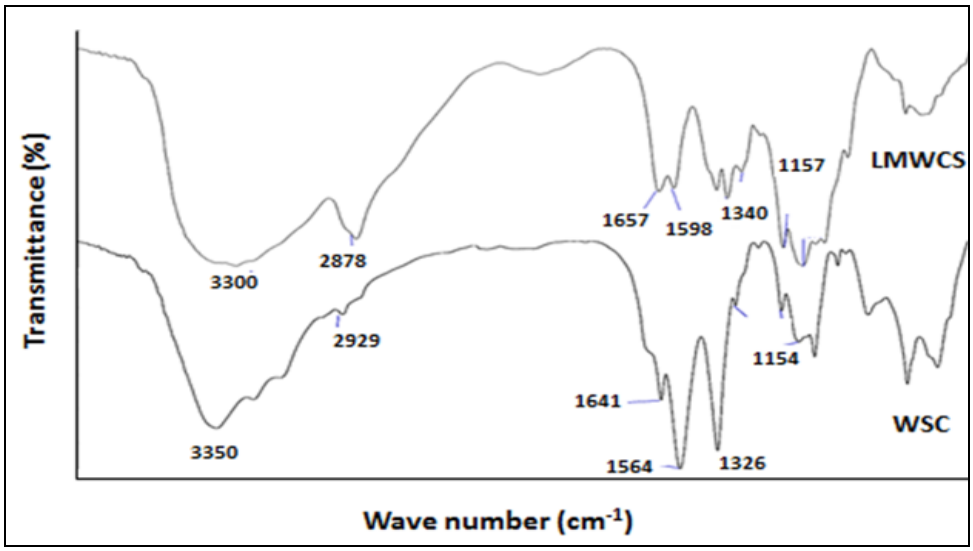

FIG. 2: FTIR SPECTRA OF WATER SOLUBLE CHITOSAN (WSC) AND LOW MOLECULAR WEIGHT CHITOSAN (LMWCS)

The results indicate that the intense characteristic peaks of WSC and LMWCS appeared at 3350 cm^{-1} and 3300 cm^{-1} represented –OH stretching and N-H stretching vibrations, respectively. The increased relative absorption intensity and relatively higher sharpness of -NH_2 in WSC spectra compared to the LMWCS is attributed to increased protonation of -NH_2 group which is expected to be due to higher degree of deacetylation in WSC resulted by enzymatic hydrolysis [26]. The characteristics peaks representing -CH stretching of -CH_2OH and -CH_3 groups on the contour of WSC and LMWCS were appeared at 2929 cm^{-1} and 2878 cm^{-1}, respectively. The absorption bands for amide I (-$CONH_2$ group) and amide III were seen at 1657cm^{-1} and 1340cm^{-1} for LMWCS.

Since the sample was presented in a solid state, amide I (more intense) and amide II may overlap, hence, amide II band would not be so noticeable for both samples. However, lower value for amide I was obtained from WSC sample, 1641 cm^{-1}; carbonyl groups had more opportunity to form stronger hydrogen bonds, as a result of higher molecules mobility. Lower wave number of amide I in WSC also affect the NH_2 bending, 1564 cm^{-1} compared to LMWCS, 1598 cm^{-1} in primary amine.

Lower value of amide III in WSC, 1326 cm^{-1}, and sharper peak at NH_2 bending suggested that it undergone an increased in degree of deacetylation. The absorption band at 1157 cm^{-1} and 1154 cm^{-1} showed slight different in term of stretching of C-O-C bridge in the chitosan. These characteristics differences in the spectra indicated that WSC synthesised with enzymatic hydrolysis showed ultra-low molecular weight and increased deacetylation degree in comparison to unmodified LMWCS.

XRD Analysis: XRD is a proven tool to yield very useful information on the degree of crystallinity of the tested sample. The XRD diffractograms of WSC and unmodified LMWCS are presented in **Fig. 3.** XRD diffractogram of WSC showed three reflections fall at 2θ=11°, 2θ=20° and 2θ=22°. The reflection fall at 2θ=11° was assigned to crystal forms I and the strongest reflection appears at 2θ=20° which corresponds to crystal forms II [27].

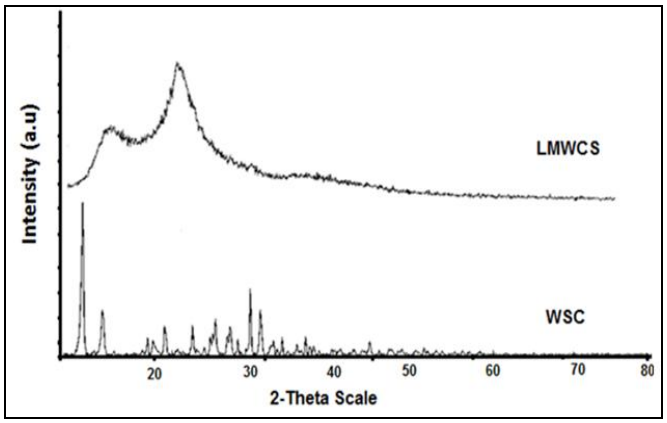

FIG. 3: XRD DIFFRACTOGRAMS OF WATER SOLUBLE CHITOSAN (WSC) AND LOW MOLECULAR WEIGHT CHITOSAN (LMWCS)

However, the unmodified LMWCS showed only one broad peak at around 2θ=20°. These results indicated that the crystallinity of WSC is increased after enzyme-hydrolytic modification compared to the unmodified LMWCS which showed amorphous nature. Although it was found that the crystallinity of LMWCS to WSC was increased, this was found not to influence solubility.

TGA analysis: Thermographs of WSC and unmodified LMWCS are shown in **Fig. 4.** The unmodified LMWCS showed a sustained weight loss starting from 110 to 280 °C due to the decomposition of polymer to low molecular weight, followed by more obvious loss of weight starting from 300 to 700 °C, which could be attributed to a complex process including dehydration of the saccharide rings, depolymerization and decomposition of the acetylated and deacetylated units of the polymer [28].

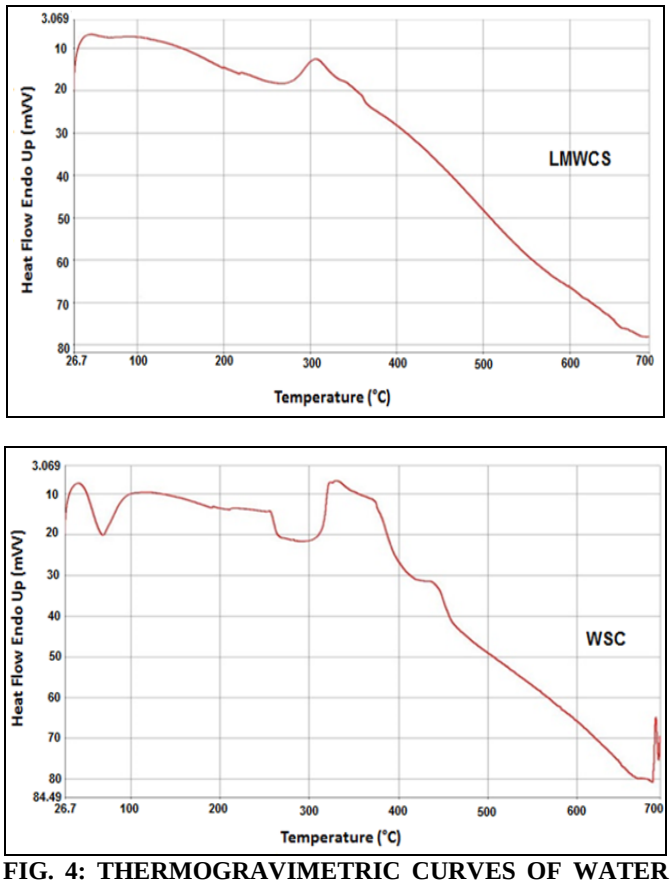

FIG. 4: THERMOGRAVIMETRIC CURVES OF WATER SOLUBLE CHITOSAN (WSC) AND LOW MOLECULAR WEIGHT CHITOSAN (LMWCS)

On the other hand, WSC showed a relatively slow weight loss from 140 to 250 °C followed by a fast obvious weight loss from 320 to 680 °C which could be due to a slow decomposition of chitosan to ultra-low molecular weight. The slower weight

loss in case of WSC indicated that it has higher thermal stability in comparison to the unmodified LMWCS and would result in more stable formulations and drug delivery systems.

Contact Angle Measurement: Contact angle (θ) is an interface that exists between a liquid and a solid surface which form an angle that described the wettability of the sample (**Fig. 5**). Prior to testing, the WSC and unmodified LMWCS samples were compressed to form discs. After the water was dropped onto the discs, the appearance of LMWCS and WSC discs were shown in **Fig. 5A** and **B**, respectively. The visual examination revealed that WSC disc showed fast spreading of water throughout the disc surface compared to the unmodified LMWCS which showed swelling of disc surface. These results showed that LMWCS is not water soluble hence drop of water onto the surface of LMWCS results into vacuum generation between the hydrophobic disc surface and the water molecules. The polymer molecules were then sucked up which eventually caused the water molecules to be stuck in between the polymer network. Contrastingly, the hydrophilic nature of WSC facilitates the easy, fast spreading of the water molecules on its surface.

Besides visual examination, contact angle (θ) was also measured for both samples. The results showed that the unmodified LMWCS yielded a bigger contact angle of 55.9° (l) and 56.6° (r) in comparison to the WSC that exhibited smaller contact angle of 44.9° (l) and 35.3° (r) (**Fig. 5**). Results from this experiment evidenced the superior wetting properties (as the contact angle was less than 45°) and hydrophilicity of hemicellulase-hydrolysed WSC, in comparison to the unmodified LMWCS.

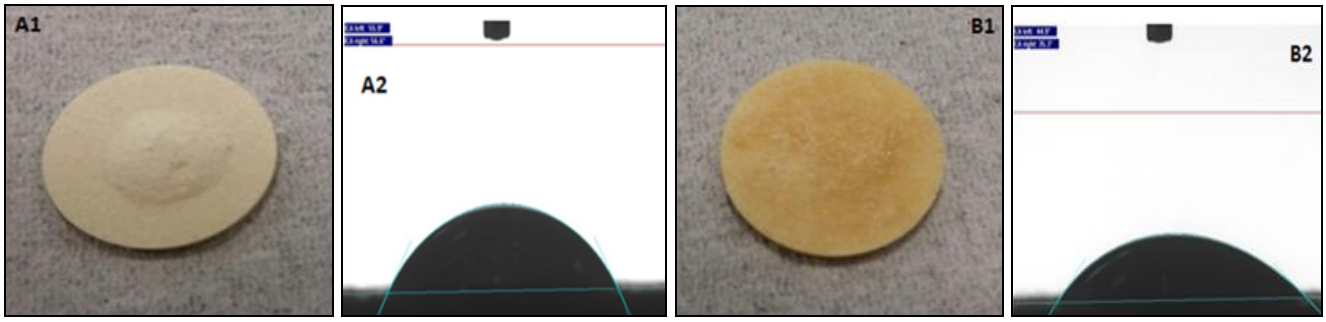

FIG. 5: MEASUREMENT OF WETTABILITY AND CONTACT ANGLE OF LOW MOLECULAR WEIGHT CHITOSAN (LMWCS) (A1, A2) ANDWATER SOLUBLE CHITOSAN (WSC) (B1, B2). RESULTS SHOWED HIGHER WETTABILITY OF WSC (Θ < 45°) COMPARED TO LMWCS (Θ > 45°)

SEM Analysis: The surface morphological analysis of WSC and LMWCS is presented in **Fig. 6**.

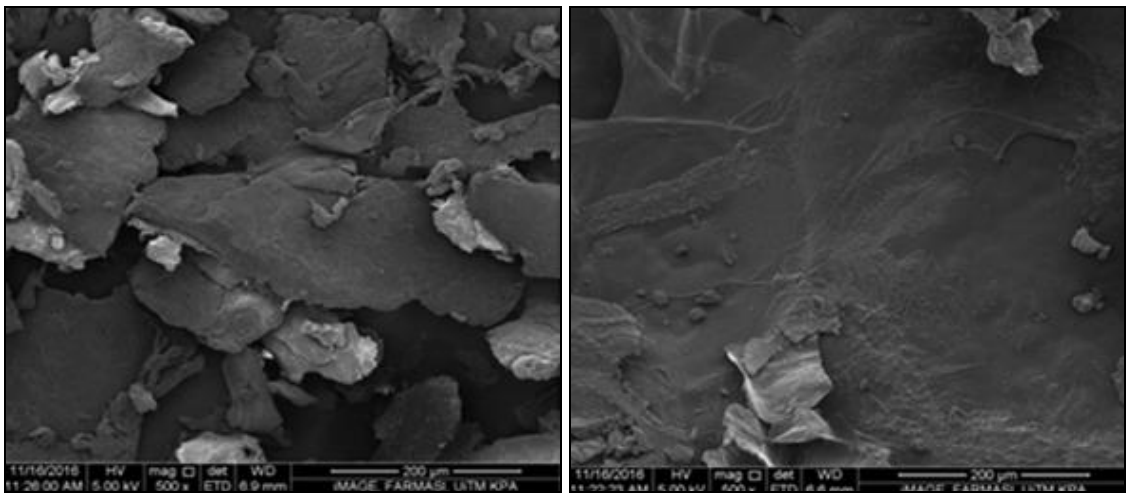

FIG. 6: SCANNING ELECTRON MICROSCOPIC (SEM) ANALYSIS OF LOW MOLECULAR WEIGHT CHITOSAN (LMWCS) ANDWATER SOLUBLE CHITOSAN (WSC) PRODUCED *VIA* ENZYMATIC HYDROLYSIS MET

The resulting micrographs revealed that WSC exhibit a smooth surface compared to the rough, more compact, and denser surface with layers of crumbling flakes in case of LMWCS. The roughness of LMWCS surface might be due to distinctly arranged microfibrillar crystalline structure that could be degraded during hemicellulase-mediated hydrolysis of LMWCS. This variability in the surface morphology of WSC and LMWCS could also be due to different intersheet or intrasheet hydrogen-bonding systems. The different arrangement of hydrogen-bonding systems and a consecutive protonation and deacetylation during the process of enzymatic hydrolysis can be the reasons for higher crystallinity and solubility of WSC in comparison to the unmodified LMWCS.

CONCLUSION: Though, numerous chemical methods have been employed for the synthesis of water soluble chitosan, these methods may change chemical nature and functionality of chitosan. In this study, hemicellulase-mediated hydrolysis was attempted to synthesise WSC with improved physicochemical characteristics including, solubility, chemical nature, contact angle, and surface morphology of water soluble chitosan. Results showed that enzymatic degradation of chitosan significantly enhanced its aqueous solubility, deacetylation degree and wettability. These findings indicated that enzymatic hydrolysis can be among the suitable methods to synthesise water soluble chitosan without effecting its physicochemical nature and biomedical applications.

ACKNOWLEDGEMENT: The authors would like to acknowledge Universiti Teknologi MARA for providing LESTARI grants (600-IRMI/ My RA 5/3/LESTARI (0133/2016). Author also would like to acknowledge Faculty of Pharmacy, Universiti Teknologi MARA, Puncak Alam campus for providing the required chemicals and reagents.

CONFLICT OF INTEREST: Nil

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How to cite this article:
Hussain Z, Sahudin S, Haliza INM, Hussain M, Sabri MLM and Ismail MA: Hemicellulase-mediated hydrolysis of chitosan: an efficient method to synthesize water soluble chitosan. Int J Pharm Sci & Res 2018; 9(1): 80-87. doi: 10.13040/IJPSR.0975-8232.9(1).80-87.

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