Journal of Applied Sciences Research, 4(12): 1755-1761, 2008

Preparation of Low Molecular Weight Chitosan by Extracellular Enzymes Produced by *Bacillus alvei*

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Abstract: Extracellular enzymes preparation (EEP) produced by *Bacillus alvei*, tentatively exhibited chitinase, chitosanase as well as cellulase and protease activities. Treatment of chitosan (30% acetylated) with these enzymes decreased their molecular weight from 88,000 to 5000 Da, after 2h. Reduction in viscosity approached zero after 2h of hydrolysis of chitosan by *B. alvei* enzymes. Enzymatic hydrolysis of chitosan by the EEP increased the solubility of chitosan hydrolyzate (89%). Optimum conditions for the enzymatic hydrolysis were pH 5.5 of chitosan solution (1%) incubated at 37°C for 2h. EEP produced by *B. alvei* possess the ability to hydrolyze chitin and a wide range of partially deacetylated chitosans. Low-molecular-weight chitosan obtained by the enzymatic hydrolysis of chitosan by EEP could be used in food industry, pharmaceutical and medical fields.

Key words: Low Molecular Weight Chitosan, extracellular enzymes, *Bacillus alvei*

INTRODUCTION

Chitin, a poorly digestible polysaccharide, is composed of β, 1-4 linkage N-acetyl-D-glucosamine residues. Chitosan, the simplest chitin derivative, is a linear polysaccharide composed of β, 1-4 linked D-glucosamine (GlcN) and N-acetyl-D-glucosamine (GlcNAc) with various composition of these two monomers[25]. Chitosan is produced chemically by deacetylation of chitin, or enzymatically by chitin-deacetylase producing microorganisms[3]. These polysaccharides, especially chitosan, have received much attention for a wide range of unique applications in food, pharmaceutical, cosmetics, agricultural, and medical fields[28,13]. Chitosan is well soluble in diluted acids, its solutions, however, even at low concentrations (0.1-0.3%) are water-insoluble and are characterized by high viscosity and fishy bitter-astringent taste, which limits their use in practice especially as food additives[30]. Chitosan oligomers with low molecular weight (1-20 kDa), however, are water-soluble and have no bitter taste. These oligomers could be obtained either by acidic[8] or by enzymatic hydrolysis[12]. In the acidic hydrolysis method a large amount of small oligomers composed of one to six glucosamine units are produced, in addition, the use of dilute acids such as 0.6M HCl would require almost 20 days to reach a molecular weight (MW) of 12,000 Da. Therefore, the enzymatic hydrolysis is preferred because it is easier to be controlled, yield large-scale production, and retain the original biological properties[16]. The most frequently used enzymes for such purposes are chitinases and chitosanases. (The action of chitinase and chitosanase enzymes on the hypothetical fragment of chitosan, as indicated by Kittur *el al.*[14], is represented in Fig. 1.) For commercial utilization, however, their use is limited due to prohibitive cost and limited availability[20]. Most recently, many enzymes including lipases, proteases, carbohydrolases, cellulases, hemicellulases and several glycanases had been found to be able to hydrolyze chitosan[24,20]. The pH and temperature optima and the dependence on the degree of acetylation (DA) and the concentration of chitosan were different for these enzymes. However, irrespective of the catalytic mechanism, the observed chitosanolytic activity for such enzymes is of interest; because they are industrially enable the production of low-molecular weight chitosan (LMWC) with low cost enzymes[24]. Fairly recently, LMWC with an average MW in the range of 5000-20000 Da were shown to possess superior biological activities compared to chitosan[21]. Jeon *et al.*[13] reported that LMWC with MW in the range 500-10000 Da had highest bactericidal activity towards pathogenic bacteria. LMWC were also shown to modulate plant resistance to disease and to stimulate murine peritoneal macrophages, killing the tumor cells, have immuno-enhancing effects and lowering cholesterol[13]. LMWC of 20 kDa were shown to prevent progression of diabetes and they had high affinity for some bacterial lipopolysaccharides[21].

Crustacean marine wastes (such as shrimp and crab shells) are a rich raw material for production of enzymes. Although this raw material is of considerable

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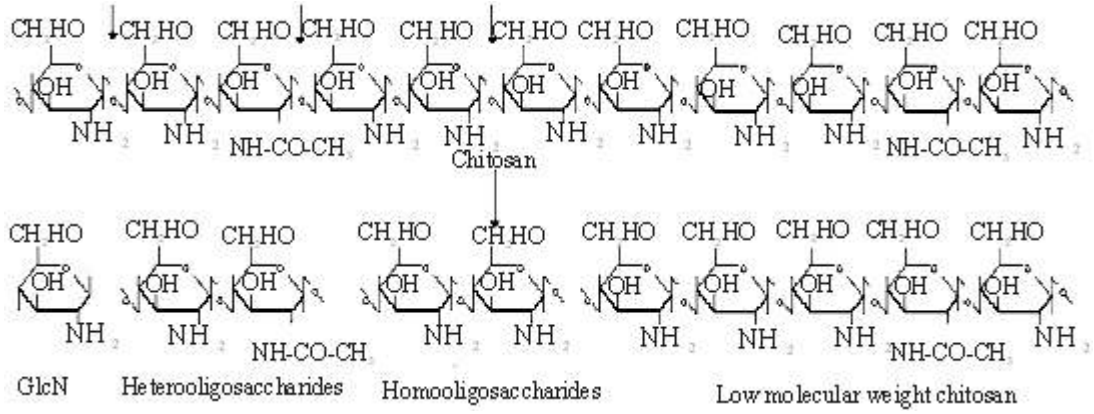

Fig. 1: Action of chitinase/chitosanase on the hypothetical fragment of chitosan

latent usefulness, currently it is not commercially used. The valuable components of shrimp shells (SS) as indicated by Nyanikova *et al.*[23] are protein, lipid, chitin and its derivatives, calcium carbonate, ash and other salts.

This work was designed to obtain LMWC from the chitosan polymer by extracellular enzymes produced by *B. alvei* strain, using SS as a low cost growth substrate. In addition, some properties of the chitosan hydrolyzates were also investigated. The feasibility of chitosan hydrolysis by other enzymes, including glycanases and proteases, was also examined in the present study.

MATERIALS AND METHODS

Organism and Growth Medium: For preparation of LMWC by *B. alvei* enzymes, the organism was grown in submerged culture on a medium containing only 30g/L shrimp shells in distilled water. Cultivation was performed in 250-ml conical flasks at 150 rpm and 30°C for 5d. The cells and remaining substrate were removed by centrifugation at 4000g for 20min. Extracellular enzymes secreted by *B. alvei* strain were determined.

Assay for Extracellular Enzymes: Measurements for proteases activity were achieved by the method of Gessesse[6] using soluble casein as a substrate and tyrosine as the standard. One unit of protease activity was defined as the amount of enzyme which releases 1µg of amino acid equivalent to tyrosine, per min under standard assay conditions. The protein content was estimated by the method reported by Lowry[17], using bovine serum albumin as a standard.

Cellulase activity was determined by the DNS (3,5-dinitrosalicylic acid) assay[18], using crystalline cellulose as a substrate and glucose as standard sugar. One unit of cellulose activity was defined as the amount of enzyme that liberates one micromole of reducing sugar per min under standard assay conditions.

The measurements of chitinolytic and chitosanolytic activities were performed according to the method of Reissing *et al.*[26]. One unit of chitinase activity is defined as the amount of enzyme that releases one micromole of reducing sugars per min at 40°C, using colloidal chitin as the substrate and with N-acetylglucosamine as standard. One unit of chitosanase is defined as the amount of enzyme that releases one micromole of reducing sugars per min at 40°C with chitosan solution as a substrate and glucosamine as the standard.

Preparation of the Chitinous Polymers: Chitin and chitosan (30% acetylated) were purchased from Sigma Chemical Co. Colloidal chitin was prepared according to the method of Monreal and Reese[19]. Chitosan solution was prepared as described by Fenton and Eveleigh[5]. Chitosans with different degrees of deacetylation (DD), by hetero- and homogenous alkaline deacetylation of crab chitin, were prepared according to the methods of Sannan *et al.*[27] and Kurita *et al.*[15]. The molecular weight (MW) of chitosan solutions was calculated by the method reported by Il'ina *et al.*[10].

Enzymatic Hydrolysis of Chitosan: Depolymerization of chitosan for the formation of the LMWC by the extracellular enzymes preparations (EEP) produced by *B. alvei* strain was performed according to the method

described by Novikov and Mukhin[22]. After preparation of a stock (200ml) solution of chitosan (1%) in a 0.2M sodium acetate buffer at pH 5.5, 50ml of the EEP was added in a conical flask. The reaction mixture was incubated at 37°C for 3h (without stirring). The degree of chitosan hydrolysis was measured by viscosity reduction of the chitosan solution using a Brookfield viscometer type LV (at 50 rpm). After measuring the reduction in viscosity of the chitosan solutions, LMWC was obtained by the method of Novikov and Mukhin[22].

Estimation of Solubility in Water: The water solubility of chitosan samples was estimated as follows. The weighed sample (10-20mg) was suspended in 10ml distilled water at 25°C for 2h with constant mixing. Soluble chitosan was removed by centrifugation at 4000g for 15min. The precipitates were thoroughly washed with ethanol, dried, and weighed. The difference between weights of samples was determined. The solubility of chitosan in water was estimated by the percent of dissolved chitosan[25].

IR Spectra of Chitosan Hydrolyzate: Chitosan and chitosan hydrolyzates were prepared in powder form. IR spectra were analyzed within the frequency range of 4000 cm^{-1} and 400 cm^{-1} using a FT-IR-FT-Raman (Nexus 670, Nicolet-Madison-WI-USA).

RESULTS AND DISCUSSION

Secretion of Extracellular Enzymes by *B. alvei:* In the previous work[1], it was reported that *B. alvei* strain possesses both chitinolytic and chitosanolytic activities.

In the present study, production of chitinase and chitosanase by *B. alvei* strain was most extensively studied using SS as a low cost substrate. Surprisingly, the organism exhibited protease, cellulase as well as chitinase and chitosanase activities. As indicated by Shahidi and Synowiecki[29], SS consists mainly of chitin (22-25%) bound to proteineous substances (34%). As shown in Fig. 2, the protease activity in the culture supernatant reached 8.0 U/ml during 48h of growth after which it was gradually decreased. On the other hand, chitinase activity reached 1.2 U/ml after 48h of growth, while chitosanase and cellulase activities increased up to 72h.

These results suggest that *B. alvei* strain, constitutively, possesses the ability to hydrolyze protein, chitin and its derivatives, that formed during the degradation of chitin. It is expected that the first step for degradation of SS components is chitin deproteinizion (by the protease secreted by *B. alvei*).

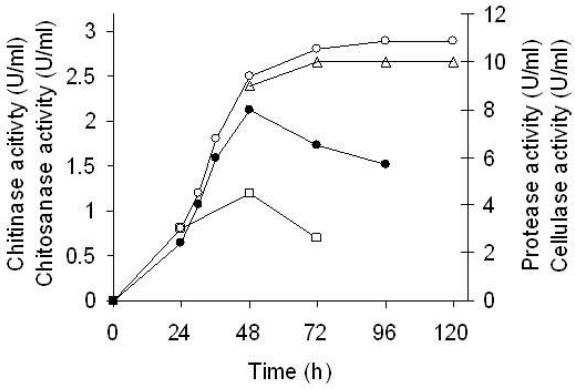

Fig. 2: Time course of degradation of SS components by extracellular enzymes secreted by B. alvei strain. Symbols: (M) protease; (G) chitinase; (") chitosanase; or (?) cellulose.

Secondly, the organism could depolymerize chitin by the produced chitinase. Reduction in chitinase activity after 48h may be due to formation of chitosan that resulted from deacetylation of chitin. Wonderful, that chitosanase and cellulase activities were simultaneously appeared and estimated after 24h of growth.

It is noteworthy that *B. alvei* strain produced both cellulase and protease when it was grown on crystalline cellulose or casein, respectively. These enzymes were found to hydrolyze a wide range of chitosan samples[24]. This result indicates that cellulase and protease produced by *B. alvei* could hydrolyze chitinous polymers. So, the EEP produced by *B. alvei* (after 48h of growth) was used for the enzymatic hydrolysis of chitosan to prepare LMWC.

Effect of the Degree of Acetylation on the Enzymatic Hydrolysis of Chitinous Polymers: The majority of microbial chitinases hydrolyze not only chitin, but also chitosan containing 20-45% N-acetyl groups[10]. On the other hand, most chitosanases isolated, so for, from different microorganisms hydrolyze only chitosan (Somacheker and Joseph, 1996). However, extracellular enzymes secreted by *B. alvei* were able to hydrolyze chitin and a wide range of chitosan samples. However, the hydrolysis of chitinous polysaccharides by *B. alvei* enzymes depends on the degree of acetylation (DA) of the substrate. As shown in Table (1), chitosan with DA of 30-50% was hydrolyzed faster compared to chitin (99% acetyl content) and fully deacetylated chitosan (1% acetyl content). Furthermore, chitosan solutions prepared under hetero-genous (CH-70) conditions was hydrolyzed faster than that prepared under homo-genous (CH-76) conditions. This result indicated that the acetyl content of chitosan molecule is an important parameter for the enzymatic hydrolysis by enzymes.

Actually, the difference between chitin and chitosan is a continual of the degree of N-acetylation of the glucosamine residue in the polymer[2]. The fully acetylated or deacetylated polymers do not occur in nature, and the only way to differentiate chitin from chitosan is to consider their respective acetyl contents symbolized degree of acetylation (DA). All the properties of chitin and chitosan depend on two fundamental parameters: the DA and degree of polymerization[4]. Both parameters influence not only the biochemical characterization of chitin and chitosan but also their biocompatibility and immunological activity.

Deacetylation of chitin (to prepare partially deacetylated chitosan) depends on three parameters that are the concentration of alkali (NaOH), temperature, and duration of the reaction[7]. As a rule, concentrated NaOH (30-50%) is used at a temperature in the range 30-120°C for 1-3h. The more severe conditions give chitosan samples, with higher degrees of deacetylation (DD), differ in their molecular weight, and chain-length distribution. Similar results would be obtained under mild condition because multiple repetitions of the reaction may cause chemical degradation of the polymer chain.

Therefore, it is incorrect to use such substrates for studying the effect of the DD on the enzymatic hydrolysis[32]. It appeared that chitosan with an acetylation content of 30-50% (Table 1) was hydrolyzed faster by *B. alvei* enzymes comparably with other highly acetylated (chitin) or highly deacetylated (chitosan) samples. These results are correlated with that reported by Il'ina and Varlamov[11], they found that chitosan with an acetylation degree of 50-54% was hydrolyzed five-to-six fold faster compared to sample of chitosan with acetylation degrees of 15 or 73% acetylation. Contrary to *B. alvei* enzymes, Qin *et al.*[25] found that chitosan samples prepared under homogenous reaction were hydrolyzed more rapidly than chitosans prepared under heterogeneous reaction.

Table 1: Effect of the degree of acetylation of chitinous polymers on the amount of reducing sugars during the enzymatic hydrolysis.

Initial polymer (DA%)	Reducing sugars (U/ml) [a]	
	Time (min)	
	10	20
Chitin (99)	0.6	1.2
Chitosan (76) [b]	0.2	0.6
Chitosan (50) [b]	0.8	2.1
Chitosan (30) [b]	1.3	2.3
Chitosan (1) [b]	0.3	0.7
Chitosan (70) [c]	0.9	1.5

[a] Colloidal chitin and chitosan solutions with different degrees of acetylation were subjected to the EEP (0.5 U/100ml) and the samples were incubated at 40°C for 20min.

[b] Chitosan solutions with different degrees of acetylation (DA) prepared by homogeneous methods.

[c] Chitosan prepared under heterogeneous conditions.

Reduction in Viscosity and MW: In fact, the viscosity loss is correlated well with reducing sugars released. As shown in Fig. 3, reduction in viscosity approached zero after 2h of incubation with EEP. Shin *et al.*[30] reported that chitosan was completely hydrolyzed by a lipase from *Rhizopus japonicus* after 10h of incubation, whereas the rate of chitosan hydrolysis by a pectinase from *A. niger* was rapid and viscosity reduction was 50% during 30 min but viscosity reduction approached nearly zero after 12h of incubation[14]. A chitinase prepared from the hepatopancreas of crab, showed higher initial rates of chitosan degradation, but it slowed down after 2h and never approached zero even after 12h[22]. This might be, probably, related to the enzyme activity which may be lost at the temperature used, or may be due to the reduction of accessible cleavage sites or increasing the proportions of bonds for which the affinity of the enzyme is lower[22].

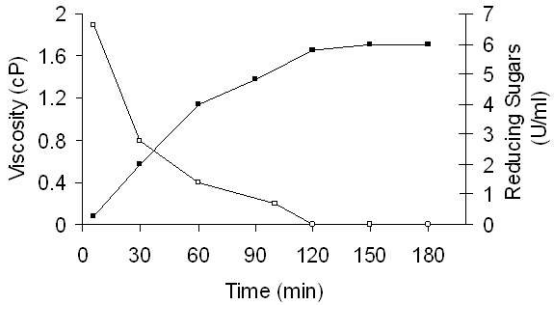

Fig. 3: Viscosity reduction of chitosan solution (30% acetylated) in presence of the EEP produced by B. alvei. Symbols: (G) reduction in viscosity; and (#) production of reducing sugars.

As for EEP produced by *B. alvei* strain, the enzymes possessed affinity for different types of linkages on the chitin and chitosan molecules as mentioned above (Table 1), and viscosity reduction approached zero after 2h (Fig. 3). Furthermore, the molecular weight of chitosan solution dropped sharply from 88,000 to 25,000 Da after 1h of contact with the enzymes, and it was around 5,000 Da after 2h (Table 2). Kittur *et al.*[14] reported that the molecular weight of chitosan dropped rapidly from 90,000 Da to 20,000 within 3h of contact with a pectinase from *A. niger*, and after 6h it was around 6,000 Da. These results suggest that LMWC could be prepared by varying the contact time with the enzyme during the time course of the polymer degradation[14].

In general, it could be suggested that the reaction of viscosity during first 30 min and the rapid hydrolysis of chitosan during 2h (Fig. 3) was a result of the synergistic and consecutive action of the novel EEP produced by *B. alvei* strain.

Table 2: Viscosity and molecular weight for chitosan and LMWC.

Sample (reaction time, h)	Viscosity	M_v (Da)
Chitosan (0) *	2.00	88,800
LMWC (1)	0.78	25,000
LMWC (2)	0.22	5,000

* Chitosan solution (1%, pH 5.5) was hydrolyzed at 37°C for 2h, using EEP.

Effect of pH on the Enzymatic Hydrolysis of Chitosan: The molecular weight of chitosan solution rapidly decreased upon contact with EEP, depending on the pH. EEP displayed an optimum pH of 5.0-6.0 (Fig. 4). Actually, measurements were done with chitosan solutions at pH 5.5. A chitosan solution of pH above 6.0 precipitates and becomes more resistant for enzymatic cleavage, subsequently the yield of LMWC was reduced. Nevertheless, strong acidity also inhibited the hydrolysis of chitosan, Fig. 4. These results are in accordance with those reported by Muzzarelli *et al.*[20], Il'ina *et al.*[9], and Qin *et al.*[25].

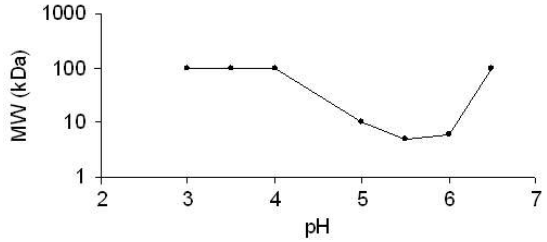

Fig. 4: pH dependence of the enzymatic hydrolysis of chitosan solution (1%), at 37°C by EEP produced by B. alvei.

Time dependent precipitate formation in the enzymatic hydrolysis of chitosan is illustrated in Fig. 5. The amount of precipitate decreased rapidly by the hydrolysis process for 2h and no precipitate was detected after 3h hydrolysis. The precipitation means that chitosan was not completely hydrolyzed and chitosan polymers were too large to be solubilized and that only very few small oligomers were produced[30].

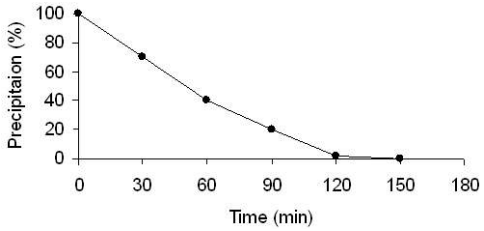

Fig. 5: Time dependentfor precipitate formation in the enzymatic hydrolysis of chitosan (pH 5.5) by EEP.

Estimation of Solubility and Sensory Evaluation: For the use of chitosan in most foods, it should be solubilized in water and should not exhibit astringent or bitter taste[30]. One of the most important characteristics of hydrolysis of chitosan solution is a decrease in the viscosity, reflecting decrease in the molecular weight and in the degree of polymerization[11]. Water solubility of the chitosan hydrolyzate products is listed in Table 3. The water solubility obviously depends on the molecular weight of chitosan. Improving the water solubility of treated chitosan is related to a reduction in its molecular weight, presumably due to the structural changes in its molecules[22].

Table 3: Solubility of the products of chitosan hydrolyzate produced by *B. alvei* extracellular enzymes.

Sample	Mw (kDa)	Solubility (%)
Initial chitosan [a]	160	0
LMWC-1 [b]	48	5
LMWC-2 [b]	26	56
LMWC-3 [b]	5	89

[a] Chitosan solution (1%, pH 5.5) was hydrolyzed at 37°C for 2h, using EEP.
[b] Chitosan hydrolyzate products.

It should be noted that the experiments were performed at a high-molecular-weight chitosan (1%). The effect of the initial chitosan concentration on the hydrolysis by EEP was studied. An increase in the initial chitosan concentration from 1-2% was followed by a rise in viscosity of the initial chitosan and, subsequently, of the end solution. Care must be taken to avoid high concentration of chitosan at which the solution was so viscous that it was not possible to measure the solubility. During hydrolysis of chitosan E/C ratio of 0.5 U/g chitosan (1%) was selected; which allowed obtaining chitosan solution with low viscosity (0.22, cP), high water solubility (89%), and minimum content of protein admixtures (0.003 g/g hydrolyzed chitosan). Il'ina *et al.*[10] reported that the protein content of the end product from chitosan hydrolyzate obtained by the enzyme preparation Celloviridine G20x was less than 0.003 g/g hydrolyzed chitosan. Generally, the products of enzymatic hydrolysis of chitosan are of particular interest (e.g. in biomedicine and as food additives). Thus, the end product should contain small amounts of protein admixtures.

Sensory characteristics of chitosan hydrolyzate (bitter-astringent taste, and fishy flavor) are very important for use in food[30]. The chitosan hydrolyzate (containing LMWC) obtained from treated chitosan by EEP was free of the fishy flavor and bitter-astringent taste.

IR Spectra of the Chitosan Hydrolyzates: In general, IR spectroscopy has been used to determine the structure of chitin or chitosan[27]. IR spectra of the original chitosan and the chitosan hydrolyzates (produced by EEP) are shown in Fig. 6. Curve *c* in

Fig. 6 shows the IR spectrum of the produced LMWC (5 kDa), which is similar to that of original chitosan (Curve *a*). Curve *b* in Fig. 6 shows the IR spectrum of LMWC (25 kDa) which is slightly differed from that of the initial chitosan (Curve *a*). Differentiations in spectrum of LMWC *b* may be due to the COO^- residue shifting from the sodium acetate buffer to amine residue of chitosan[30].

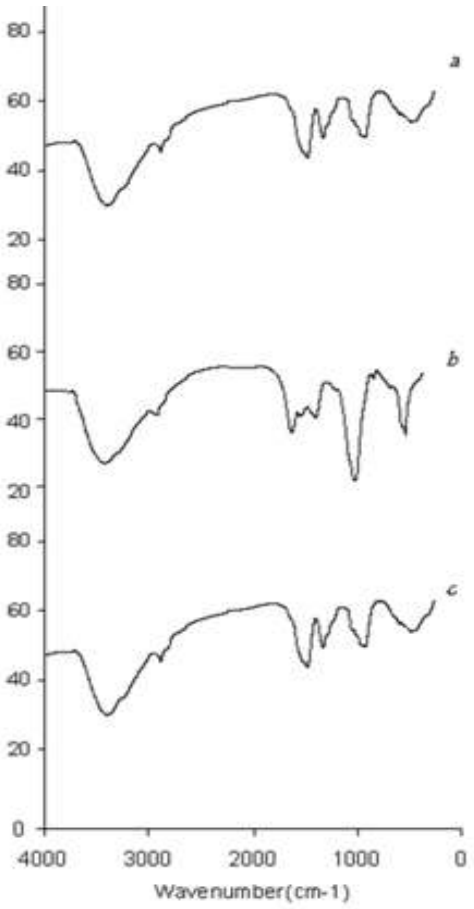

Fig. 6: IR spectra of initial chitosan (a), and its hydrolyzates (b, c).

Further studies on the solubility of the chitosan hydrolyzates produced by *B. alvei* enzymes and their relation to structural changes after hydrolysis are required and will be studied to explore their application in food and other pharmaceutical and medical fields.

Conclusion: Partially N-acetylated chitosan was hydrolyzed with the cheap, efficient EEP produced by *B. alvei*. The optimum temperature for the enzymatic hydrolysis was around 40°C and the optimum pH was 5.5. Low-molecular-weight water-soluble chitosan (20 and 5 kDa) obtained using EEP from *B. alvei* will be of special interest to the food industry as well as other industrial fields because of their solubility in water and over a wide range of pH values from 6 to 9.

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