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Extracellular Metabolites Produced by a Novel Strain, *Bacillus alvei* NRC-14: 4. Antimicrobial Activity of Chitosan Oligosaccharides

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ABSTRACT

Degradation of chitosan by specific and/or non-specific enzymes resulted in the production of chitosan oligosaccharides (COSs) which effectively confirmed to be inhibitor agents. These bioactive chitosan oligosaccharidesare used mainly for medical applications due to their specific biological activities. In the present study, flaked chitosan was used along with sugarcane bagasse as carbon sources for production of COSs by a novel bacterial strain, *Bacillus alvei* NRC-14.Thisstrain is,constitutively, able to producecarbohydrate-active enzymes.The enzymes activity increased from 1.7 U/ml, in a minimal chitosan medium, to 4.6 U/ml by optimizing the culture conditions using sugarcane bagasse as carbon source. The crude culture was found to contain a variety of enzymes,i.e. chitosanase, glucanase, cellulase as well as chitobiase and high amounts of cellobiase. These enzymes efficiently degraded flaked chitosan to COSs. Three chitosan oligosaccharides with different molecular weights were obtained; signed CH-76, CH-25, and CH-10. Antimicrobial activity of native chitosan and these COSs against bacteria and fungi was shown to be closely dependent on the molecular weight, degree of deacetylation as well as the pH and the strain tested. Recently, COSs are extensively demonstrated to be used as inhibitor agents, functional food additives and as preservatives.
Keywords: metabolites, chitosan, sugarcane bagasse, chitooligosaccharides, antimicrobial activity.

1. INTRODUCTION

Chitin and chitosan are natural polysaccharides with a chemical structure similar to that of cellulose, and their differences are in functional groups at the C-2 positions of their constituent sugars, i.e., the hydroxyl (–OH), acetamido ($NH\text{-}CO\text{-}CH_3$) , and amino groups ($\text{-}NH_2$) in cellulose, chitin, and chitosan, respectively (Nogawa*et al.* 1998). Chitosan, a natural nontoxic biopolymer produced by the deacetylation of chitin, is a major component of the cell wall of the zygomycete fungi, e.g., *Mucorales, Rhizopus, Fusarium*,and*Absedia.*Chitosan in nature is not fully deacetylated; it is a copolymer of glucosamine and *N*-acetylglucosamineresidues, which usually distributed randomly in the chitosan molecule. Chitosan is insoluble in water, but it dissolvesin diluted solutions of organic acids (such as acetic and citric acids) and diluted inorganic acids (such as hydrochloric acid). Chitosan contains three types of reactive functional groups: amino, acetamido, andhydroxyl groups. These groups are the main reason for the physiochemical properties of chitosan. Chitosan demonstrates antifungal, antiviral, andantibacterial properties that make it a favorable option for biomedical and pharmaceutical fields (Chirkov 2002,Dash *et al.* 2011). In addition, chitosan has been used in food industry as a stabilizer and thickener, as a preservative, and asa clearing agent (Jeon*et al.* 2000). Moreover, chelation of heavy metalsand flocculation properties of chitosan in industry have been established as well as a good drug delivery and in cosmetics (Huang *et al.* 2009, Dai *et al*. 2012).However, the large molecular weight (MW), poor solubility in water, and highly viscous nature of chitosan restrict its use in the *in vivo* systemsas a functional food (Jeon*et al.* 2000).

Depolymerization of chitosan to prepare chitooligosaccharides(COSs) and low molecular weight chitosan (LMWC)has received growing attention.COSs are molecules with an average molecular weight below 3.9 KDa, whereas LMWC is ranging between 3.9 to 20 KDa. These chitosan derivatives are water-soluble and could be easily digested and absorbed in the human intestinal to reduce cholesterol, blood pressure, blood sugar, and blood lipid level in serum and liver (Xia 2003; Wang *et al.* 2009)and also display antimicrobial, antitumor, and antioxidant activities (Berit*et al*. 2010). LMWC and COSs are also introduced into a variety of biomedical fields including wound dressings and drug delivery systems, biochemical industries, and in food industries(Riberio*et al*. 2009). Chitosan can be depolymerized using specific enzymes such as chitinases and chitosanases, or non-specific enzymes such as cellulases, lipases, pectinase, α-amylase, and protease (Jeon*et al.* 2000).Enzymatic methods for preparation of COSs have received great interest due to their safety, simplicity, and could be easily controlled. Studies on the biological activities of chitosan have been increasing, as and enzymatic products have different structures and physiochemical properties, which may result in novel bioactivities or

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novel findings of new bioactive compounds (Wang *et al.* 2007).

The use of renewablewastes to produce biologically valuableproducts and to minimize environmental wastes is a challenge for current research and development. Sugarcane industry and mill generate huge amounts of sugarcane bagasse. Since biodegradation of this waste is very slow, accumulation of large quantities has become a major problem. Production of COSsby chitosanases, using this renewable waste, is a dual-purpose opportunity. Chemical analysisof sugarcane bagasseand some other lignocellulose waste-materials are represented in Table 1(Nigam *et al.* 2009).There are various reports dealt with production of enzymes, single cell protein, ethanol, using raw or pre-treated bagasse in submerged fermentation. In our previous studies, a novel strain identified as *Bacillus alvei* NRC-14, was found to produce carbohydrate-active enzymes such as chitinases, chitosanases, ß-glucanases as well as cellulases and xylanase. This bacterial strain is particularly interesting because of the variety of extracellular metabolites it produces. Moreover, it has adapted special metabolic pathways to survive in extreme conditions and so has better capacity to produce special bioactive compounds.Thepresent studydealt withthe production of COSs from flaked chitosan using specific (chitosanase) and non-specific (cellulose and glucanase) enzymes.Antimicrobial activity of these COSs was also investigated. To the best of our knowledge, production of COSswiththe aid of specific and non-specific enzymes by thenewly isolated*B. alvei* NRC-14 has never been reported.

Table 1. Main components of lignocellulose waste materials (Nigam *et al*. 2009).

Lignocellulose waste	Cellulose (wt %)	Hemicellulose (wt %)	Lignin (wt %)
Barley straw	33.8	21.9	13.8
Corn cobs	33.7	31.9	6.1
Corn stalks	35.0	16.8	7.0
Cotton stalks	58.5	14.4	21.5
Rice straw	36.2	19.0	9.9
Soy stalks	34.5	24.8	19.8
Wheat straw	32.9	24.0	8.9
Sugarcane bagasse	40.0	27.0	10.0

MATERIALS AND METHODS

Microorganism and culture conditions

The strain,*B. alvei* NRC-14,was grown in medium containing (g/L): 6.0, flaked chitosan; 1.5, $(NH_4)_2SO_4$; 0.5, $MgSO_4.7H_2O$; and 4.0, sugarcane bagasse with pH adjusted to 5.5. After autoclaving,cultivation of the strain was performed in 250-ml flasks under shaking conditions (130 rpm) at 30^{o}C for 5 days. At intervals, culturesamples were centrifuged at 7000 x g for 15 min, andthe resultant supernatant was used for detection of enzymesand glucosamine.

Pretreatment of agricultural residues

The lignocellulosic materials used in this study were all obtained locally. These waste materials were first dried, copped into small pieces, ground into smaller particles in a hammer mill, and finally separated by a 0.45mm (40 mesh) sieve (Gao*et al.* 2008). No chemical pretreatment was done for any of the waste materials used. All lignocellulosic wastes were used as carbon sources at a concentration of 0.4%.

Determination of enzymes activity

Chitosanase activity was determined using soluble chitosan. Chitobiase activity was determined using *p*-nitrophenyl-*B*-D-glucosaminide (Nogawa*et al.* 1998). Cellulaseand CMCase activities were detected using Avicel as a substrate (Lin *et al.* 2009). Cellubiase activity was determined using *p*-nitrophenyl-*B*-D-glucopyranoside as described by Tao *et al.* (2010). Xylanase activity was determined using 1% xylan solution as a substrate (Tao *et al.* 2010).The amount of reducing sugars was determined using the dinitrosalicylic reagent (Miller 1959).

Production of chitosan oligosaccharides

After incubation period, the culture broth was centrifuged at 7000 x g to remove solid residues. The supernatant obtained was heated in a boiling water bath for 15 minutes to denature the enzymes (Phakapob*et al.* 2008). The culture supernatant, containing COSs, was objected for selective fractionationto separate COSs according to the method reported by (Juan and Pierri 2005).

Determination of Molecular Weight

The Molecular Weight (MW) of chitosan oligosaccharides were detected with the aid of viscosity according to the calculation method reported byXie*et al.* (2011).The intrinsic viscosity of chitosan solution (η) was detected, and its viscosity average molecular weight (Mη) was calculated according to the following formula:

$[\eta] = 11.0 \times 10^{-3} M\eta^{0.85}$

Determination of degree of deacetylation

Samples were assayed for infrared spectroscopy. Degree of deacetylation (DDA) of products was calculated by the following formula:

$$DDA\ \% = \frac{1.33 - A_{1655}/A_{3450}}{1.33} \times 100$$

Where A_{1655} was the absorption peak of amideat the wave length of 1655 cm^{-1}, and $_{A3450}$ was the absorption peak of NH at the wavelength of 3450 cm^{-1}.

Assay for antimicrobial activity

Antimicrobial activity of three COS sampleswith different molecular weights in addition tohigh molecular weight chitosan with degree of deacetylation (DDA) of 10 and 90, i.e. CH-10 and CH-90,were examined against*Eschericia coli, Staphyloccocusaureus,Pseudomonas aeruginosa, Aspergillusniger,* and *Aspergillusflavus,* as the test microorganisms. These samples were added at initial of cultivation and the mixtures were incubated with shaking at 37^{o}C for bacteria and 30^{o}C for fungi, for 48 hrs, after which the growth was measured at A_{610}nm (Liu *et al.* 2006). The antimicrobial activity of COSs was also determined using the agar plate technique (Monarul*et al.* 2011).Suspended cultures of the test organisms (0.1 ml) were firmly seeded over the nutrient agar plates.

IR-spectra

The IR-spectra of the produced chitosan derivatives were detected. The spectrum of the sample was recorded on the spectrophotometer over a wave number range 4000-400 cm^{-1}using a FT-IR- Raman (Nexus 670, Nicolet-Madison-WI-USA).

RESULTS AND DISCUSSION

Production of enzymes fromlignocellulosic wastes

Besides to serve as low-cost raw materials for the production of important metabolites and enzymes, reuse of the lignocellulose in fermentation processes is an environment friendly method of waste management. Since these wastes are rich in sugars, which are easily assimilated by microorganisms, they are very appropriate for use as raw materials in the production of industrially relevant compounds by fermentation. The lignocellulosicwastes,namely: rice straw,wheat straw, wheat stalks, corn stalks, and sugarcane bagasse were used for production of enzymes by *B. alvei* NRC-14. As represented in Table 2, sugarcane bagasseis the most carbon source yielded chitosanase, cellulase, CMCase and ß-1,4glucanase, followed by rice straw and corn stalks.So, sugarcane bagasse was used as carbon source in the present study for production of non-specific enzymes.Lignocellulose in the form of agricultural and agro-industrial wastes is accumulated in large quantities every year. These materials; composed mainly of cellulose, hemicellulose, and lignin, are extensively used for growth of microorganisms and production of valuable compounds such as ethanol, food additives, organic acids, enzymes, and others as an alternative to solve environmental problems caused by their disposal.

Table 2.Enzymes produced by *B. alvei* NRC-14 (U/ml) using various agricultural residues.

Lignocellulose waste	Chitosanase	Cellulase	CMCase	Glucanase	Xylanase
Rice straw	2.67	21.30	33.20	4.70	10.90
Wheat straw	1.89	14.11	18.97	0.89	4.65
Wheat stalks	2.24	16.90	18.35	1.96	4.78
Corn stalks	2.63	17.83	21.08	4.86	7.43
Sugarcane bagasse	3.21	31.09	34.77	5.75	16.08

Time course of enzymes production

Whensugarcane bagasse was used alongwith flaked chitosan as carbon sources:firstly, chitosanase activity started and reached a maximum before 2 days of growth,after which it gradually decreased (Fig. 1, A). Chitinase and detectable amounts of *D*-glucosaminidase were estimated after 3 days (Fig. 1,A). Activity of CMCase and cellulasereached a maximum at the second and third days, respectively (Fig. 1, B),whereas xylanase activity increased after 2 day. In addition, activity of chitobiaseand cellubiase started after 3 days(data not shown).On other hand, detectable amounts of glucosamine were observed at 30hrs of growth which gradually increased up to 70hrs (Fig. 2), at which negligible amounts of COSs were detected. Being a linear copolymer of glucosamine (GA) and *N*-acetylglucosamine (NAG), chitosan contained four types of linkages, viz., GA-GA, NAG-GA, GA-NAG and NAG-NAG. Class I chitosanase

cleavesthe first two types of linkages: class II cleaves only the first, whereas class III cleaves both first and the third type of linkages. NAG-NAG linkage (exists in chitin only) will be cleaved only by chitinases. Chitinase A from *B. alvei* NRC-14 (Abdel-Aziz *et al.* 2008) exhibited specificities for hydrolyzing powdered and colloidal chitin (NAG-NAG linkage), chitosan with different degrees of deacetylation, chitobiose, NAG, as well as cellulose, CM-cellulose and xylan. These results indicate that, *B. alvei* NRC-14 possesses an enzymatic system that has an affinity for the ß–1,4 linkages in a variety of carbohydrates.

The solubility of polymers such as chitin, chitosan, crystalline cellulose, xylan, and granular starch is poor. This is the main reason why such natural polymers can't be effectively degraded by microorganisms. Solubility of chitosan isrelated to the protonated amino groups ($-NH^{+3}$) in the polymer chain. Being ß–glycanases and has different affinities for the ß–1,4 bonds, cellulaseand glucanaseshowed specificity for chitosan. Thus, the synergistic and consecutive action of the crude enzymes from strain NRC-14 has contributed to the effectiveness of chitosan degradation, leading to the high yield of COSs.This, together with the capability of the strain to producechitobiase and cellobiase, suggested its potential use as a promising agent for biocontrol and in the industrial field.

Production ofchitosan oligosaccharides

During incubation period, reducing sugars reached a maximum after less than 48hrsof growth (Fig. 2), whereas amaximum yield of GA was occurred at a time period exceed 60hrs of growth. This result indicates that, by controlling the growth course,both COSs and GA could be easily obtained. The MW of COSs obtained was about76kDa, 25kDa, and 10kDa (signed CH-76, CH-25, and CH-10).

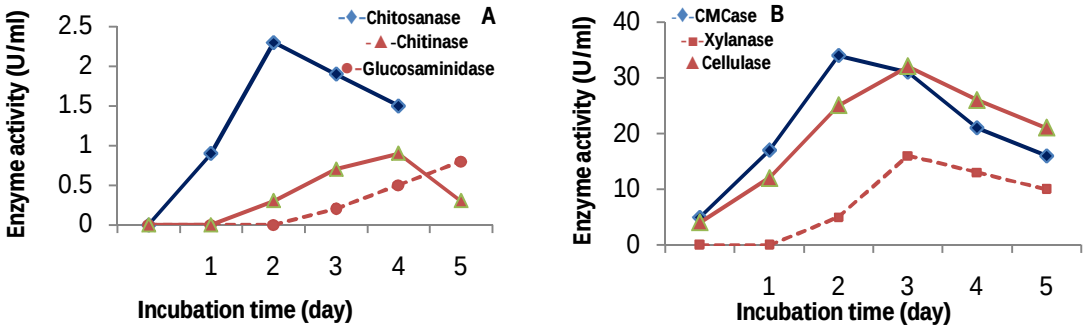

Fig. 1.Enzymes produced by *Bacillus alvei* NRC-14 using sugarcane bagasse (4 g/L) and chitosan (6 g/L) as carbon sources.Cultures were incubated under shaking condition at 30°C for 5 days.

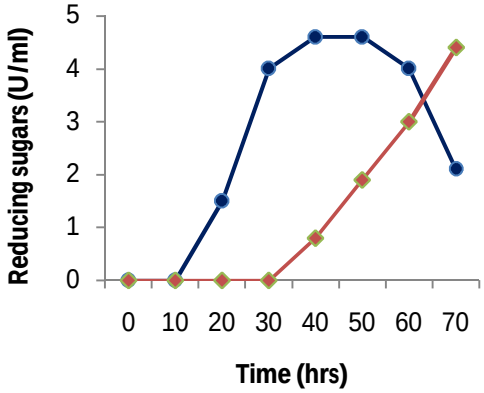

Fig. 2. Reduction in reducing sugars content (●) and enhancement of glucosamine (♦) formation by*Bacillus alvei* NRC-14 at 30°C using sugarcane bagasse along with flaked chitosan.

Antimicrobial effect of chitosan oligosaccharides

The main factors affecting the antimicrobial activity of chitosan oligosaccharides are MW, DDA,pH and the strain tested. Effect of chitosan and its oligosaccharides was evaluated against *E. coli*, *S. aureus*, *P. aeruginosa*, *A. niger*, and *A. flavus*. The inhibitory effect of low MW chitosan [CH-10] is shown in Fig. 3.On other hand, growth inhibitory effect of chitosan and its oligosaccharides (Fig. 4) showed that, growth of *E. coli* wasaffected by the chitosan sample CH-76and

native chitosan with higher MW and different DDA (ChiT-10 and ChiT-90), whereas *S. aureus*and *P. aeruginosa* were mostly affected by COSs with low MW (CH-10, CH-25, and CH-76). Both *A. niger*and *A. flavus*were highly affected by all chitosan samples, irrespective of MW or DDA (Fig. 4).

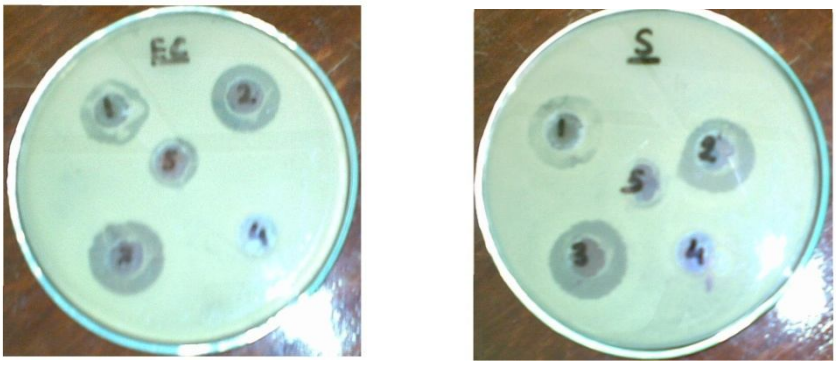

Fig.3. Inhibitory effect of Low molecular-weight chitosan (CH-10) upon cell growth of *E. coli*;(Left image)and *S. aureus*(Right image)as indicated by clear zones, after 24hrs of growth**.**

Effect of chitosan and its oligosaccharides was evaluated against *E. coli*, *S. aureus*, *P. aeruginosa* , *A. niger*, and *A. flavus*. The strain, *S. aureus*, can cause skin infections, such as pimples, impetigo, boils, cellulitis folliculitis, carbuncles, scald skin syndrome, and abscesses, life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis. It is still one of the five most common causes of nosocomial infections (Munarol*et al.* 2011). Regarding *E. coli*, itis one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection, traveler's diarrhoea, and other clinical infections such as neonatal meningitis and pneumonia (Monarul*et al.* 2011, Sahoo*et al.* 2012). *P. aeruginosa*,are opportunistic pathogens often associated with infections of the urinary tract, respiratory system, soft tissue, bone and joint, gastrointestinal infections, dermatitis, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, cancer and AIDS (Ozumba 2003).*A. niger* is one of the most common causes of human otomycosis (fungal ear infections) which can cause pain andsevere cases, damage to the ear canal and tympanic membrane. Some strains of *A. niger*have been reported to produce potent mycotoxins called ochratoxins (Schuster *et al.* 2002). *A. flavus*are also well knownto cause human diseases; it is the second most common agent of aspergillosis, the first being *Aspergillusfumigats. A. flavus* may invade arteries of the lung or brain and cause infarction. Neutropenia predisposes to aspergillus infection. *A. flavus* also produces anaflatoxin which is one of the aetiological agents for hepatocellular carcinoma (Klich 2007).

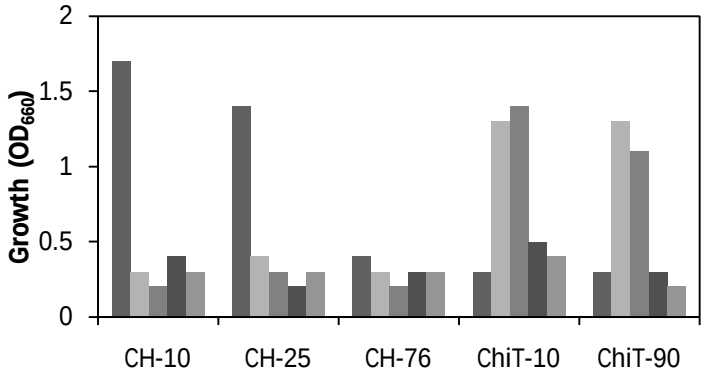

Fig. 4. Growth inhibitory effect of chitosan and its derivatives; CH-10, CH-25, CH-76, ChiT-10 and ChiT-90 upon cells of *E. coli* (■), *S. aureus*(■), *P. aeruginosa*(■), *A. niger*(■),and *A. flavus* (■).

Effect of pH on theantimicrobial activity of chitosan and its oligosaccharides was also investigated. The antimicrobial activity of a chitosan oligosaccharide [CH-10]was tested over the pH range of 3.5–5.5. As shown in Fig. 5, the tested fungi were inhibited over a pH range of 3.5–5.5, whereas *E. coli*, *S. aureus*and *P. aeruginosa*were mostly affected by lower pH values (3.5–4.5). These results are in accordance with previous reports (Xia *et al.* 2010).

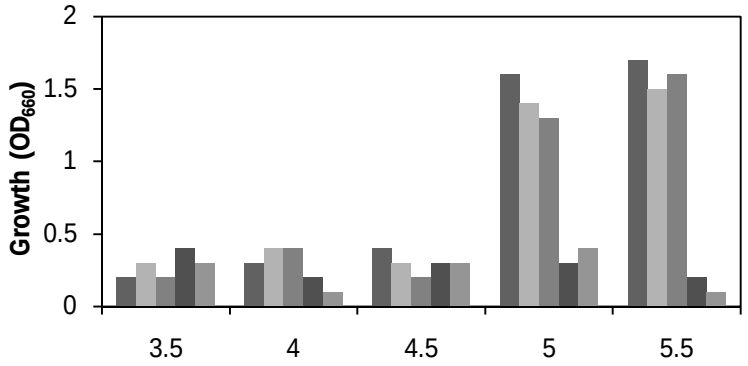

Fig. 5. Effect of different pH values on the antimicrobial effect of Low molecular-weight chitosan (CH-10) upon cell growth of the tested microbes: *E. coli* (■), *S. aureus*(■), *P. aeruginosa*(■), *A. niger*(■),and *A. flavus* (■).

Different hypothesis have been proposed to elucidate the mechanism of antimicrobial activity of chitosan. Chitosan contains three types of reactive functional groups, an amino/acetamido group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. The amino contents are the main reason for the differences between their structures and physicochemical properties which are all correlated with their chelation, flocculation and biological functions. The mode of action of cationic antimicrobial agents is,however, widely believed to be due to interacting with and disrupting the wall/ membrane structure (Helander*et al.* 2003).In Gram-positive bacteria, the cell membrane is covered by a cell wall made up of 30–40 layers of peptidoglycans, which contain *N*-acetylglucosamine, *N*-acetylmuramicacid as well as D- and L-amino acids including isoglutamateand teichoic acid, to which the positively charged amino groups of chitosan oligosaccharides/GlcN can bind, resulting in distortion, disruption, and exposure of cell membrane to osmotic shock and exudation of the cytoplasmic contents. Gram-negative bacteria, on other hand, contain an outer membrane wherein lipopolysaccharide and proteins are held together by electrostatic interactions with bivalent metal ions, one to two layers of peptidoglycans and a cell membrane (containing lipid bilayer, trans-membrane proteins and inner/outer membrane proteins). The negatively charged O-specific antigenic oligosaccharide-repeating units of the *E. coli* lipopolysaccharide form ionic-type of binding with the amino groups of chitosan oligosaccharides, thus blocking the nutrient flow with concomitant bacterial death due to depletion of the nutrients (Kumar *et al*. 2005). It is suggested that, high content of amino groups in chitosan molecules leads to a decrease in the inhibitoryeffect.

Differences in inhibitory effect values are reported in many studiesincluded chitosan, especially when various MWs are tested,in attempts to find the one that exerts the highest antibacterialeffect. Jeon*et al*. (2001) reported that a 10kDa COS is theminimum MW required for inhibition.Zheng and Zhu(2003) reported that COS with MWof 5kDa yielded the highest inhibition effect over *E. coli*, whereas a 305kDa fraction had the highest effect against *S. aureus*. No *et al*. (2002) reported that themost effective MW against those two bacteria is 470kDa. In this study, 10-KDaand 25 KDa COSsshowed antimicrobial activity against bacteria and fungi except for *E. coli*, while COS76-kDaand high MW chitosan (ChiT-10 and ChiT-90) resulted in an inhibitory effect upon *E. coli*and fungal cells. These differences are probably accounted for by the distinct experimental conditions used by different authors, viz., the MWrange, the degree of deacetylation, the concentration, the final pH, as well as the bacteria and chitosan sources, and the solvents employed (Fernands*et al.* 2011).The interaction between chitosan and microbial cells,proposed to be mediated by the electrostatic forces between the protonated NH^{3+} groups and the negative residues,is most probably occurred by competing with Ca^{2+} for electro-negative sites on the membrane surface (Xia*et al.* 2010). This electrostatic interaction results in twofold interference: i) by promoting changes in the properties of membrane wall permeability; and ii) by the hydrolysis of the peptidoglycans in the cell wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase).

IR-spectra

As seen in Fig. 6, the FT-IR spectrum of the chitosansamples showed the presence of absorption bands at 2924 cm^{-1}(lift arrows) corresponding to the carbonyl group (Xia *et al*.2010).The absorption bandsaround 1577-1634 cm^{-1}(middle arrows)were measured as characteristic bands for chitosan and its derivatives (Muzzarelli*et al*. 2004).The absorption peaks observed around 899 cm^{-1}(right arrows)are generally known to be typical characteristics of aminosugar derivatives (Suh*et al.* 1997).

Prospects and limitations for application of chitosan and its derivatives are well documented.In the food industry, chitosan (edible chitosan, more than 83% DDA) and COSs have been used as dietary food additives and functional factors

due to their antimicrobial, hypocholesterolemic and immune-stimulating effects as well as drug carriers. Many commercial functional products have been available, such as chitosan capsules and COS capsules in China and Norway (Xia *et al*. 2010). Edible chitosan biofilm has also been prepared for food storage, utilizing its antimicrobial activity. The drug-deliverycharacteristics of chitosan and its derivatives has made them popular both in functional food and medicine as they can deliver drugs or functional factors to the target and control release. However, it should be noted that chitosan with different structures showed different biological activities, and not all the biological activities are found in one kind of chitosan. Each special type of bioactive chitosan should be developed for its potential application. In fact, it is hard to explain exactly how these molecules exert their activities (Xia *et al.* 2010). Therefore, future research should be directed towards understanding their molecular-level details which may provide insights into the unknown biochemical functions of chitosan and COSs as well as help to accelerate their future application.

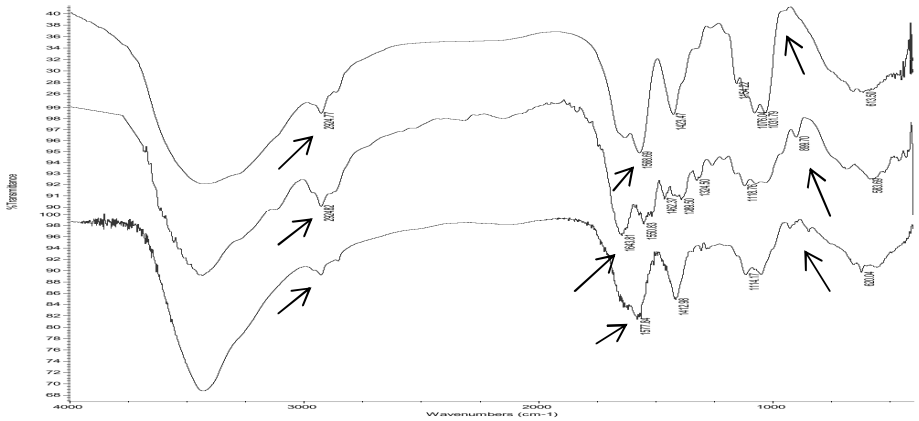

Fig. 6. IR-spectra of COSs produced by *Bacillus alvei* NRC-14: CH-10 (above curve), CH-25 (middle curve) and CH-76 (bottom curve).

For use of chitosan and COSs as a functional food, it should be free of fishy flavor,astringent and bitter taste and must be water-soluble. Foods or food products that can provide health benefits, beyond their standard nutritional requirements, are referred to as functional foods and nutraceuticals. These food products are formulated to contain significant levels of components that have been demonstrated to reduce the risk of disease and potentially improve overall health. Some of health benefits regarding functional foods are represented in Table 3. Improving water solubility of the treated chitosan is related to a reduction of its MW, presumably, due to the structural changes in its molecules (Novikov and Mukhin 2003).Moreover, COSs with low MW retained over a wide range of pH values.High-water solubility of COSs may probably be attributed to the decrease of intermolecular interactions, such as van der Waals forces and hydrogen bonds (Qin *et al*. 2004).

Table 3. Health benefits of some marketed functional foods (Katan and Roos 2004).

Ingredient	Example product	Health benefits
Probiotic bacteria	Yoghurt and milk products	Improve the gut functions and enhance immunity
Omega-3 and Omega-6 fatty acids	Fish, tuna, shrimp, fruits, nut, eggs, cereals, some vegetables	Immune defense, heart protection, eyesight development
Isoflavones (phyto-estrogens), Flavonoides	Soy products, Green tea, mint, chocolate, lettuce, fresh vegetables.	Reduce osteoporosis, prevent cardiovascular disease, improve cardiovascular health
Plant phytosterols and phytosanols	Yoghurt, margarine, barley	Lower LDL cholesterol
Conjugated linoleic acid	Yoghurt, dairy products, meat, food supplements	Weight loss, cancer prevention
Herbs and herbal extracts	Green tea, drinks, cereals	Energy enhancement, memory improvement, weight loss
Non-starch polysaccharides and other fibers	Cereals, soy-been, fresh fruits	Improve mineral absorption, lower cholesterol, weight loss, improve gut health, potential anticancer effect
Antioxidants, vitamins, and minerals	Fresh fruits and vegetables, cereals (folic acid)	Reduce oxidative stress, prevent cells oxidation

Conclusion

In thepresent work,lignocellulose wastes were exploited for production of enzymes. Using sugarcane bagasse along with flaked chitosan, specific and non-specific enzymes were secreted bythe strain in the culture medium and resulted in production ofCOSs and glucosamine by time-controlling. The crude enzymes degraded the large molecular chain of

chitosan rapidly under mild conditions, decrease the molecular weight, and tended to be an efficacious approach to produceCOSsand glucosamine.The produced COSs obviously exhibited antimicrobial activity towards a number of danger microbes.From a commercial standpoint, specific and non-specific enzymes produced by strain *Bacillus alvei*NRC-14 has many beneficial characteristics, such as constitutive production, high productivity, high specific activity, strong enzyme stability, production of COSs with high antimicrobial activity against bacteria and fungi.These antimicrobial compounds are of great value regarding to their utilization as a natural food additive and food preservative, since they are biocompatible, non-toxic, water-soluble, and consumption safe.

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