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Microbiological Chitosan: Potential Application as Anticariogenic Agent

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1. Introduction

Dental caries is the most prevalent oral disease that affects a significant part of the world population, especially in less developed countries. It is universally accepted that the dental caries is a chronic and multifactorial disease [1-3]. The permanence of the bacterial plaque on the tooth surface will lead to loss of minerals constituents of the dental enamel promoting the installation of the carie disease. The carious lesion is characterized by the tooth structure (hydroxyapatite) demineralization by the production of organic acids, such as lactic acid, resulting from bacterial (dental biofilm) metabolism. This results in loss of calcium and phosphate ions which subsequently diffuse out of the tooth. In this complex process, the microorganisms, particularly *Streptococcus* species, have an important role in its etiology [3-5].
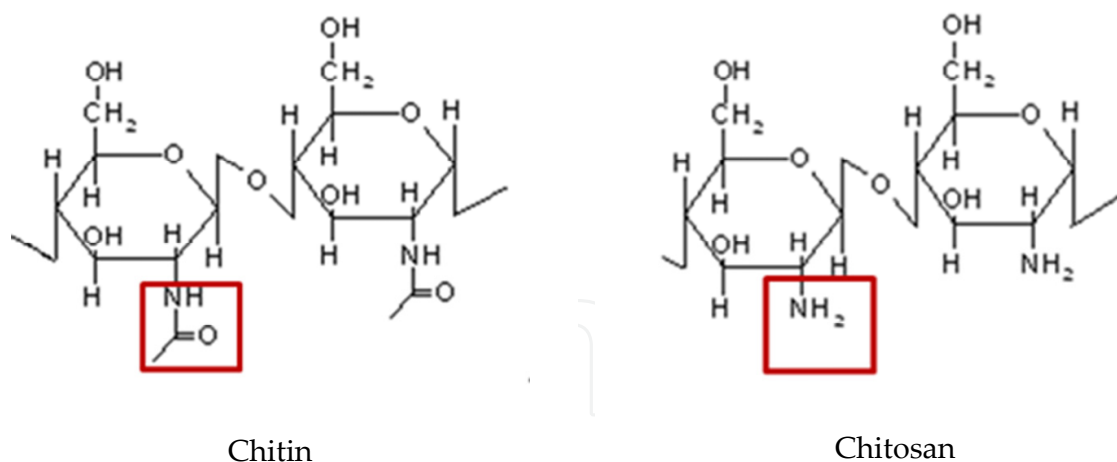
Many oral *Streptococcus* in the presence of carbohydrate produce organics acids and insoluble glucans which serve as binding sites for bacteria on the tooth surface, forming the biofilm. The sucrose plays an important role in carie development, influencing on biofilm acidogenicity and cariogenic microflora. The high cariogenicity of dental plaque formed in the presence of sucrose can be mainly explained by the high concentration of insoluble glucans on its matrix, the low inorganic concentration and its protein composition may have some contribution [6, 7].

Specifics microorganisms are associated with dental plaque formation, development and maturation. The *Streptococcus mutans* is a member of the oral microbial community which plays a key role in formation of cariogenic biofilms. The key factors of *S. mutans* cariogenic are the production of a great variety of carbohydrates, which generate low pH and cause the consequent demineralization of the tooth enamel [3, 8-10].

Recent developments in the areas of biomaterials devices have resulted in a number of advances in the searches of natural substances which may inhibit the dental plaque formation and/or the hydroxyapatite demineralization [11, 12]. In particular, this research is focused on novel macromolecules and biocompatible materials for use in clinical applications. Chitosan is a non-toxic and natural polysaccharide, which have many biological applications, mainly as antimicrobial agent [12].

2. Chitosan: General considerations

Chitosan is a natural co-polymers of chitin, composed by units of 2-amino-2-desoxi-D-glycopyranose and of 2-acetamide-2-desoxi-D-glycopyranose interconnected by glycosidic bonds β-1,4 in variable proportions. The first type of units is frequently present in chitosan. This polymer is naturally found in the cell wall of fungi, mainly in the Mucorales order [5, 13, 14]. Chitosan is formed by the chitin deacetylation, and the group N-acetyl can be suffer several degrees of deacetylation (Figure 1). Chitosan is characterized according to its deacetylation level and molar mass, once such features may influence the degradability and in the polysaccharide hydrolysis [15, 16]. According to the medium acetylation level (AL), chitosan may be obtained with physical-chemical properties differentiated regarding the solubility parameters, pKa and viscosity [17, 19]. It is difficult to obtain chitosan with high deacetylation level as due long process of isolation, and the degradation of the polymer also increases [5, 15].

Chitin   Chitosan

Figure 1. Chemical structure of Chitin and Chitosan (Source: authors)

The synthesis of chitin from fungi and crustaceans differs significantly; however, steps of enzymatic machinery for biosynthesis and catalytic regulation are similar. In crustaceans is initiated by conversion of glucose 6-phosphate N-acetylglucosamine-1-phosphate, involving a series of stages that require several enzymatic reactions. N-acetylglucosamine-1-phosphate reacts with UTP forming the UDP N-acetylglucosamine, which finally transfers N-acetylglucosamine for the polymerization of chitin chains in a process mediated by the

enzyme chitin synthase and Mg $^{+2}$ ion as catalyst, resulting in the formation of a long chain of subunits monosaccharide's linked by β-1 → 4. Chitosan is thus obtained by deacetylation of chitin by chemical treatment with NaOH at high temperature [20].

Fungi the chitin and chitosan synthesis simultaneously were occurred. The synthesis of chitin is highly compartmentalized. The enzyme chitin synthase is the zymogen form and distributed in specific regions of the cell surface, vesicles in specialized, chitosome. The macromolecular assembly starts out of the cytoplasm, where the protease enzyme acts on the cell surface activating the zymogenes. In this way the UDP N-acetylglucosamine is produced from glucose, and chitin synthase catalyzes the transfer of N-acetylglucosamine for polymerization chain forming chitin. The chitosan present in cell walls of certain fungi (Mucorales) is formed from the deacetylation (chitin deacetylase) chain chitin source for the biosynthesis of chitin. The regulations of the synthesis of chitin and chitosan are determined by the spatial organization of the synthesis of chitin in the cell surface [20, 21].

Crustacean chitosan is inconsistent in its physical–-chemical properties due to the variability in raw materials, the harshness of the isolation and conversion processes, the caustic effects of the chemicals used in the isolation process, and variability in the levels of deacetylation and protein contamination [22-24].

The use of biomass from fungi have demonstrated a great advantages, such as: independence of seasonal factor, wide scale production, simultaneous extraction of chitin and chitosan, extraction process is simple and cheap resulting in reduction of the time and cost required for production, and also absence of proteins contamination, mainly the proteins that could cause allergy reactions in individuals with shellfish allergies. However, to optimize the production of chitin and chitosan from fungi, it's usually used complex or synthetics cultures media, which are expensive. It's becomes necessary to obtain economic culture media that promote the growth of fungi and stimulate the production of the polymers [25-32].

In order to obtain alternative sources of nutrients and low cost several research projects are being conducted. Table 1 shows the use of various synthetic media and low cost alternative media used for growth of fungi of the order Mucorales, and production of chitin and chitosan. The content of chitin and chitosan from fungal cell wall varies among different species and growth conditions. Many studies have been performed to verify the possibility of using the biomass of fungi, especially Mucorales, class Zygomycetes, as an alternative source of chitin and chitosan. Many of these studies test simple models to verify the production of chitin and chitosan, ie, the approach adopted experiments using only one variable at a time during the fermentation, eg after cultivation, agitation, pH, temperature and concentration of nutrients. However, the literature report in recent years the scientific interest to reduce the numbers of tests and increase the accuracy of the results has been increased. Therefore, multivariate approach, using factorial design, allows the observation of the synergistic effect between the independent variables, since all variables are considered simultaneously, resulting in the final optimize conditions [30-34].

Microorganism	Substrate	Biomass (g.L^{-1})	Chitin (mg.g^{-1})	Chitosan (mg.g^{-1})	Reference
Mucor circinelloides	Yam bean	20.70	500	64	33
Cunninghamella elegans	Yam bean	24.30	440	66	32
Cunninghamella elegans	Hesseltine and Anderson added of 5%NaCl and 6%glucose	24.40	388	70	29
Cunninghamella bertholletiae	Sugar cane juice	7.70	-	128	30
Aspergillus niger	Potato Dextrose Broth	9.00	-	107	34
Lentinus edodes	Potato Dextrose Broth	1.4	-	33	34
Zygosaccharomyces rouxii	Yeast Malt Extract Broth	4.4	-	36	34
Candida albicans	Yeast Malt Extract Broth	1.8	-	44	34
Rhizomucor miehei	Sabouraod dextrose (SDB)	4.1	-	13.67	24

- Data not shown

Table 1. Chitin and chitosan production by Mucorales strains compared with *Cunninghamella cirnelloides* using yam bean as substrate.

3. Properties, antimicrobial and toxicity of fungi chitosan

Chitosan has numerous applications in several areas, mainly biomedical and pharmaceutical fields, due to its specific properties Among their properties we highlight the excellent biocompatibility; almost any toxicity to human beings and animals; high bioactivity; biodegradability; reactivity of the group amino deacetylated; selective permeability; polyelectrolyte action; antimicrobial activity; ability to form gel and film; chelation ability and absorptive capacity [36-38]. These peculiar properties provide a variety of applications to the chitosan, such as: drug carrier of controlled release [36], anti-bacterial [37, 39] and anti-acid [38]; inhibition of the bacterial plaque formation and decalcification of dental enamel [2, 10]; promotes the osteogenesis [40]; and promotes the healing of ulcers and lesions [41, 42].

The applicability of chitosan is related to their physical-chemical properties considering the different sources (crustaceans, fungi, and mollusk's) and different processes for extraction and purification cause alterations in the degree of deacetylation, molecular weight, thermal stability and degree of crystallinity of the chitosan. Various derivatives of chitosan differ of the degree of deacetylation, molar weight, arrangement of residual N-acetyl groups in the chain considering the reaction of -NH 2 group at the carbon 2 (C2) or unspecific -OH group in position 3 carbons or 6 (C3 or C6) of the polymer with other functional groups are reported in the literature [15].

Chitosan is a weak base insoluble in water but soluble in dilute aqueous solutions of various acids, the most widely used is acetic acid [43]. The acid solubility is explained by the protonation of the free amino group, characteristic in the chitosan *in natura*, which change to NH_2 to NH_3^+, whereas in alkaline condition, the hydro solubility is due to the formation of carboxylate, from the introduced carboxylic group [19, 44]. The possibility to obtain a variety of polymer derivatives with differences solubility, thermal stability, reactivity with other substances and specificity regarding the binding site, providing several biological applications of the chitosan [15]. Some applications of the chitosan, it is highligh it's the use in the pharmaceutical industry, more specifically related to dental clinic [43].

Chitosan has a recognized antimicrobial activity, being this, one of the main properties of the polysaccharide. Several researchers demonstrated that this polysaccharide has antimicrobial action in a great variety of microorganisms, included gram-positive bacteria and various species of yeast [21, 45]. In the literature is described that chitosan acts in the cellular wall of the microorganism modifying the electric potential of the cellular membrane [46]. This polysaccharide also acts potentiating other inhibition drugs, as the chlorhexidine gel, once it increases the drug permanence time action place [47, 48].

In reference [39] the authors report that chitosan has demonstrated low toxicity and the resistance development have not occurred. The antimicrobial action of the chitosan and its derivatives suffers influence from factors, which depending on the performed role may be classified in four main categories: 1. Microbial factors as species, age of the cell); 2. Intrinsic factors of the chitosan as: positive charge density, molecular weight, hydrophobic and hydrophilic characteristics, chelation capacity; 3. Physical state factors (soluble and solid state), and 4. Environmental factors (pH, ionic forces, temperature, and time).

The antimicrobial action mechanism of the chitosan is not yet fully elucidated, being several mechanisms are suggested by the literature. Some authors suggested the amino groups of the chitosan when in contact with physiological fluids are protonated and bind to anionic groups of the microorganisms, resulting in the agglutination of the microbial cells, and growth inhibition [49, 50]. On the other hand, reference [49] report that when interacting with the bacterial cell, the chitosan, promotes displacement of Ca^{++} of the anionic sites of the membrane, damaging them. Another postulate is the interaction between the positive load of the chitosan and the negative load of the microbial cell wall, because it causes the rupture and loss of important intracellular constituent of the microorganism life. Chitosan with low molecular weight penetrates in the cell and is linked to the microorganism DNA inhibiting the transcription and consequently the translation, whereas the chitosan of high molecular weight acts as a chelate agent, binding to the cell membrane [16].

Authors in reference [47] investigated the relation between antimicrobial activity of the chitosan and the characteristics of the cellular wall of bacteria. They verified that the chitosan is antibacterial agent more efficient to Gram-negative bacteria due the composition of phospholipids and carboxylic acids of the bacterial cellular wall. These results suggest that the effects of the chitosan are distinct in the two types of bacteria: in the case of the gram-positive, the hypothesis is that chitosan of high molecular mass may form films

around the cell that inhibit the absorption of nutrients, while chitosan of low molecular mass penetrates more easily in gram-negative bacteria, causing riots in the metabolism of these microorganisms.

Chitosan from *M. circineloides* was obtained as described the literature [33]. The chitosan with different molecular weight was obtained by methods of extraction. Hight molecular weight of chitosan was obtained by methodology described by Stamford et al [32], and Fai et al.[33], and low molecular weight of chitosan by methodology described by Hu et al.[54]. The degree of deacetylation for chitosan was determined by infrared spectroscopy, the molecular weights by viscosity described by Stamford et al [32] and Fai et al.[33]. However, the chitosan with low molecular weight showed antimicrobial activity to Gram positive and negative bacteria than chitosan of high molecular weight (Table 2)

Bacteria	Minimum inhibitory concentration (mg/ml)		Minimum bactericidal concentration (mg/mL)	
	Chitosan		Chitosan	
	ChLMW	ChHMW	ChLMW	ChHMW
S. aureus	1.25	2.5	2.5	5.0
E. coli	1.25	2.5	2.5	5.0
P. aeroginosa	0.625	1.25	1.5	5.0
S. mutans	0.625	1.25	1.5	5.0

Table 2. Minimum Inhibitory Concentration (mg.mL^{-1}) of chitosan from *Mucor circineloides* with different molecular weight: Low molecular weight chitosan with 3.2×10^3 g/mol (ChLMW) and higher molecular weight chitosan 2.72×10^5 g/mol (ChHMW), against pathogenic bacteria

Studies *in vitro* were carried out in the Microbiology laboratory of UFPB to determine the Minimum Inhibitory Concentration (CIM) and the Minimum Bactericidal Concentration (MBC) of chitosan from different origins, and varying the parameters deacetylation degree and molecular weight to oral Streptococcus species. The results showed greater influence of the molecular weight of the polymer on antibacterial activity. The low molecular weight of chitosan have shown higher antimicrobial activity to CIM and CBM, when compared with chitosan gel (soluble in acetic acid 1%), and with higher molecular weight, as described in the Table 3.

In addition, chick embryo chorioallantoic membrane (CAM), is a rapid and inexpensive method for determination of tissue reactions to biomaterials, and was used to evaluate the chitosan toxicity and physiological compatibility. Positive control was used as sodium lauryl sulfate 1%. The method allows prediction of the potential irritation by chitosan was studied.

Fertile hen's eggs at 10º days of incubation at 37°C, obtained from Guaraves Guarabira Aves Ltda, were used in the tests. Five eggs were used for each chitosan solution assayed. On day 10 of incubation, the egg shell above the air space was removed. The exposed membrane was moistened with a drop of 0.9% physiological saline and the saline was removed, uncovering the CAM. An aliquot of 200 μl of chitosan solution was applied on the CAM. Signs of vasoconstriction, hemorrhage and coagulation for 5 minutes were observed to

evaluate the potential for irritation according to the method of HET-CAM. Thus, biocompatibility of chitosan was evaluated including the search for signs of inflammation, edema or neovascularization [55].

Chitosan	Streptococcus species							
	S. mutans		S. sanguis		S. oralis		S. mitis	
	CIM	CBM	CIM	CBM	CIM	CBM	CIM	CBM
Crustaceans 65DA% 3,5 x 10⁶	1.25	5.0	1.25	7.5	1.25	5.0	1.25	5.0
Crustaceaus 90DA% 3,5 x 10⁶	1.25	2.5	1.25	5.0	1.25	2.5	1.25	2.5
Crustaceans 90DA% 3,1 x 10⁴	0.625	1.25	0.625	2.5	0.625	1.25	0.625	1.25
Fungi 80% DA 2,72x 10⁶	1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5
Fungi 90% DA 2,72x 10⁶	1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5
Fungi 90% DA 2,3x 10⁴	0.625	1.25	0.625	1.25	0.625	1.25	0.625	1.25

Table 3. Studies *in vitro* performed by Microbiology laboratory, UFPB (João Pessoa-PB, Brasil) demonstrating the Minimum Inhibitory Concentration (CIM) and Minimum Bactericidal Concentration (CBM) of the chitosan (derivatives from different origins, 80 and 90% of deacetylation degree (DA), molecular weight (MW) g/mL and solubility) to *Streptococcus* species

Assays	Chmw	Clmw	SLS 1%
Vasoconstriction	0.0±0.0	0.0±0.0	6.0 ±1.0
Hemorrhage	0.0±0.0	0.0±0.0	48 ±3.0
Coagulation	0.0±0.0	0.0±0.0	63 ±3.0
Irritation potential	0.0±0.0	0.0±0.0	17,74 ± 0,4

Non-irritating: 0-0.9 ; slightly irritating: 1-4.9; Irritating: 5-8.9 and Severely irritating: 9-21

Table 4. Test of chitosan to high (Chmw) and low (Clmw) molecular weight against vasoconstriction, hemorrhage and coagulation. Positive control: sodium lauryl sulfate 1% (SLS1%).

Assays	Inflammation		edema		neovascularization	
	Chmw	Clmw	Chmw	Clmw	Chmw	Clmw
1	0.0	0.0	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	0.0	0.0

Table 5. Test of chitosan to high (ChHMW) and low (ChLMW) molecular weight for inflammation, edema and neovascularization.

4. Anticariogenic effects of chitosan

Experiments were performed in the Microbiology Laboratory-Nucleus of Research in Environmental Sciences- UNICAP (Recife, PE, Brazil) regarding the safety concentration for the dilution of fungi chitosan in acetic acid. The mechanisms of chitosan from crabs and fungi to inhibit the tooth colonization by *S. mutans*, *S. sanguis*, *S. mitis* and *S. oralis* were evaluated through the adherence test of chitosan to dental and bacteria surface, results showed in Figure 2. Chitosan from crabs and fungi, in all concentration tested, decreased the adsorption of *Streptococcus* strains to dental enamel, reduced the bacteria cell wall hydrophobicity and decreased the glucan production by bacteria. However, chitosan from fungi was more efficient than chitosan from crabs for the tree parameters studied.

Figure 2A shows the decrease of bacteria adsorption to dental enamel in the presence of chitosan from crabs and fungi, in all concentrations studied. Chitosan demonstrated best performance at the concentration of 2mg/mL for *S. mutans* and of 3mg/mL for *S. sanguis*, *S. mitis* and S*. oralis*. These results are in agreement with the one obtained by [8], which studied the effect of chitosan from crabs of low molecular weight in the adsorption of *S. mutans*, *S. sanguis* and *S. oralis* to the commercial hydroxyapatite.
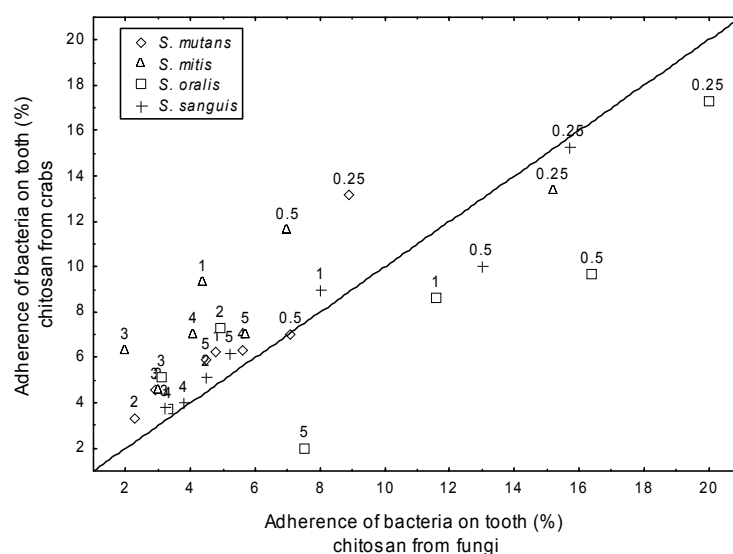
Researchers [9, 10] investigated, "in vivo", the activity of a chitosan mouthrinse, respectively of 1% and 0.5%, and verified significant reduction of dental plaque formation. The authors reported that chitosan might be altering of the electrostatic interaction between the bacterial cell surface in saliva and tooth pellicle surface. The electrostatic interaction is usually repulsive due to the fact that inature both bacteria and the pellicle surface are predominantly negatively charged. The chitosan chains attach themselves to the negatively charged bacterial cell surface by means of their positively charged groups. If these chains are of a sufficient length to bind more than one cell, bridges are formed between bacterial cells. As soon as the bridging becomes effectives flocs are formed, and the bacteria cannot colonize the tooth surface.

In literature [8-10] is reported that aggregating oral bacteria may reduce their adherence to tooth surface. The polycationic nature of chitosan might reduce the initial bacterial adherence onto the teeth surfaces, at least in part, by generating bacterial aggregation. There have also been suggestions that bacterial aggregates are removed more easily from the oral cavity than individual bacterial cells.
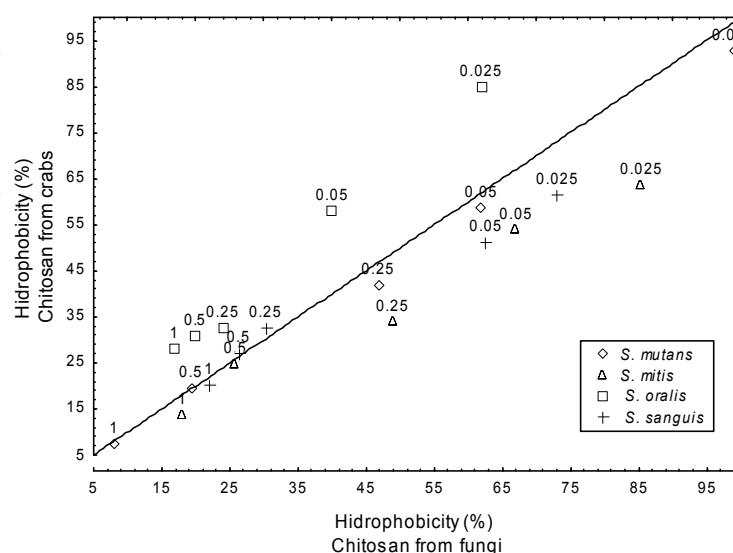
To verified the influence of chitosan, sublethal concentration, to modification in bacterial cell surface, it was evaluated the affinity of chitosan to xylene of bacteria grown in the presence or absence of chitosan sub-MICs, through hydrophobicity tests (Figure 2B), and for sucrose catabolization, through extracellular glucan production by bacteria strains (Figure 2C). The results in figure 2B indicates that increasing concentration of chitosan in bacterial suspension caused a successive decrease of the bacteria cell wall hydrophobicity, being more evident for the *S. mutans* strain. These results are supported by findings of [8,9]. These authors suggested that chitosan induced a successive decrease in cell hydrophobicity, and that surface hydrophobicity is related to adherence ability of bacteria. Therefore, the

inhibiting effects of chitosan may depend, at least, partially, on alterations of bacterial cell surface hydrophobicity expression.
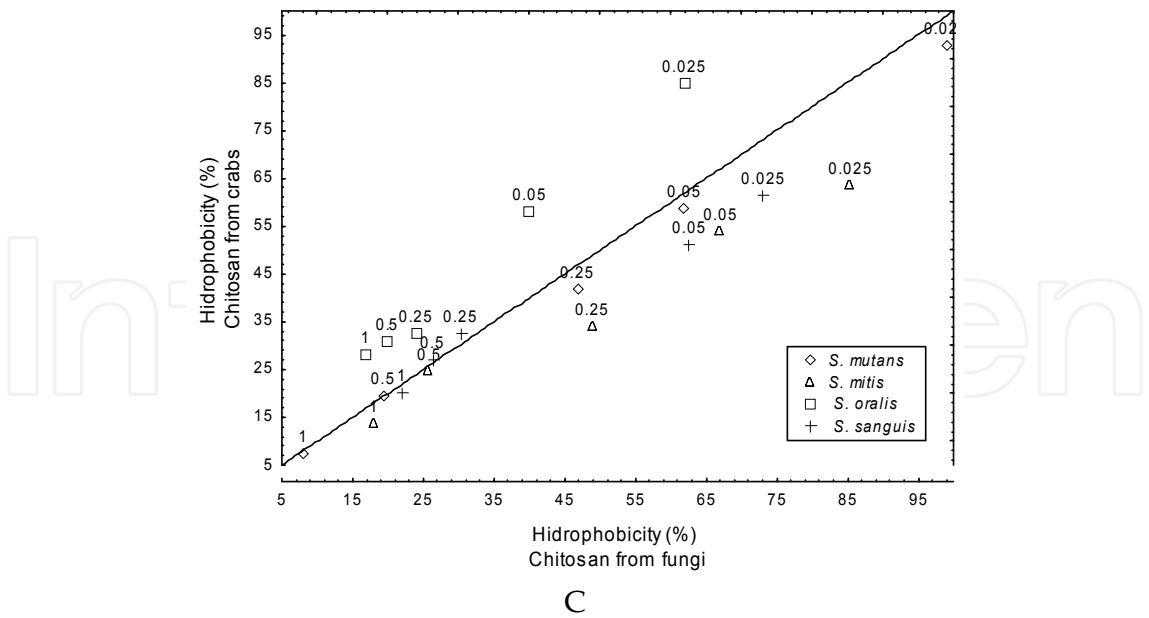
In figure 2C is observed a decreasing of extracellular glucan production by bacteria strain in presence of sucrose, with increasing chitosan concentration. The ionic interaction between the cation, according to [61,62], from chitosan (amine group) and anionics parts of bacteria cell wall (phospholipids and carboxylic acids) can form a membrane polymer, which prevents nutrients from entering the cell. Since chitosan could adsorb the electronegative substance, this polymer can promote cell flocculate, and disturbs the physiological activities of the bacteria and kill them. Authors [63] reported that chitosan interact with the electronegative bacterial cell surface resulting in displacement of Ca^{++} from anionic membrane sites, resulting in a changing the electric potential of the bacteria surface.
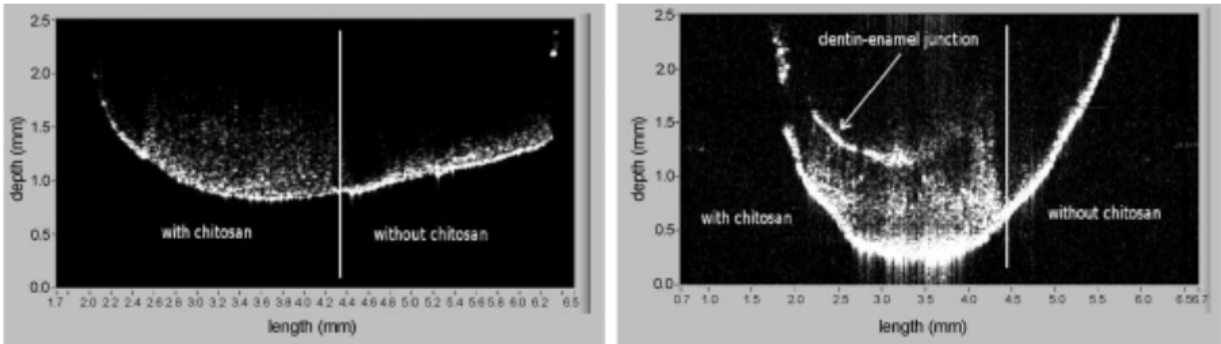

A

B

C

Figure 2. Diagram of stratification dispersion of chitosan from fungi and crabs activity: inhibition of bacteria adsorption on dental enamel (A); hydrophobicity of bacteria surface (B) and glucan production by bacteria (C).

Experiments were conducted in the department of chemistry's fundamental UFPE verified the penetration depth of chitosan in teeth enamel for two specimens by Optical Coherence Tomography (OCT) images. Chitosan was applied only to half of the teeth surface. The image in the left was obtained for a chitosan concentration of 1.25mg/ml. it is clearly seen the penetration of chitosan as indicated by the brighter area (caused by light scattering) in the figure, only in the region where chitosan may act as a mechanical barrier for the acid penetration in the enamel, which would explain its effect in the demineralization inhibition [2].

Sources: [2]

Figure 3. Optical Coherence Tomography (OCT) images of specimens treated with chitosan at 1.25mg/mL (left) and 5.0mg/mL (right)

6. Conclusion

The use of chitosan in different formulations, such as toothpaste (Chitodent®), mouthwash solution and chewing gum, is mentioned in literature [8-10]. In all forms the chitosan has

shown antibacterial action for bacteria belonging to the group *Streptococcus*, inhibits the growth and adherence of cariogenic bacteria and the desmineralization process of dental enamel in vitro, and stimulates salivation, *in vivo*. The results confirm a high biotechnological potential for chitosan from both fungi and crab sources as a cariostatic and anticariogenic agent, suggesting their application as dentistry biomaterial for prevention and therapeutic of dental carie.

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