



Optimal conditions for levan biopolymer production and its use in the synthesis of bactericidal levan-ZnO nanocomposite

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Abstract

With the ever-increasing resistance of pathogens to various antibiotics, it has become critically important to find novel biocompatible antibacterial agents. This research focuses on the optimization of the biological synthesis of levan biopolymer using the Taguchi method in order to produce levan-ZnO nanocomposite. Attempts have been made to synthesize this nanocomposite to improve the antibacterial activity of ZnO nanoparticles. Optimization of growth conditions led to the improved levan-producing capabilities of the *Zymomonas mobilis* PTCC 1718 strain (57 g/l). Molten salt and *in situ* methods were applied for the synthesis of ZnO nanoparticles and levan-ZnO nanocomposite, respectively. Ultraviolet-visible (UV-vis) spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM) confirmed the formation of levan biopolymer, ZnO nanoparticles, and levan-ZnO nanocomposite. Antibacterial analysis showed that the formation of nanocomposite improved the antibacterial activity of ZnO nanoparticles. The present study has demonstrated that levan-ZnO nanocomposite characterized by the capability to destroy Gram-positive and Gram-negative microorganisms might be utilized as an antibacterial agent in the medical, pharmaceutical, dentistry, and food industries.

Key words: nanocomposite, levan biopolymer, zinc oxide, antibacterial, *Zymomonas mobilis*, Taguchi method

Introduction

One of the main challenges facing the global community today is discovering effective therapies for cancer (Mozaffari et al., 2016; Mozaffari et al., 2017), chronic pains (Sharifi et al., 2017), chronic kidney disease (No-mani et al., 2016), autoimmune diseases (Mozaffari et al., 2018), and microbial infections (Taran et al., 2018; Imani and Safaei, 2019). Microbial infections are historically considered as major pathogenic factors causing mortality around the world (Li and Webster, 2018). Nowadays, due to the increased bacterial resistance to antibiotics, it has become necessary to find innovative ways of eliminating bacteria. Applying nanoparticle based materials on the grounds of their features and bene-

fits as antimicrobial compounds has received increasing attention (Beyth et al., 2015; Safaei and Taran, 2017).

Levan is an exopolysaccharide with a molecular weight of 107 Da; it is made up of about 60000 units of fructose. The main chain is formed by β -links (2,6); the branches are generated by β -1,2 bindings (Alegre et al., 2005; Arvidson et al., 2006). Levan biopolymers are obtained in different ways, of which one is its production by a wide range of bacteria during the enzymatic transfructosylation reaction. *Zymomonas mobilis* is an important bacterium in the microbial production of levan biopolymer. It is a Gram-negative rod-shaped bacterium with a length of 2-6 μm and a width of 1-1.4 μm ; it is non-sporulating.

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Appropriate viscosity and stability in the face of pH adjustments, water and oil solubility, solubility in organic solvents, heat resistance (melting point temperature of 225 °C), high water holding capacity and capacity to hold chemical materials, as well as favorable biological characteristics, has made levan a unique polymer that is indispensable in many fields. Levan as an emulsifier, stabilizer, an encapsulating agent, and a concentration factor is used in several sectors of medical, pharmaceutical, and food industries (Bekers et al., 2005; Srikanth et al., 2015). It serves as an anti-inflammatory and immunomodulatory agent, blood plasma substitute, antitumor, antioxidant, and anti-AIDS compound in medication (Abdel-Fattah et al., 2012; Silbir et al., 2014). Levan biopolymer is also applied for drug delivery, improving the flavor of tablets and solubility of capsules, and generating a more rapid medicinal response (Srikanth et al., 2015). The exopolysaccharide is critically important in the industry because of its sweetening and antibacterial characteristics. Byun et al (2014) reported the antibacterial characteristics of levan compounds with low molecular weight. The widespread use of levan in different industries has transformed it into a multipurpose polymer (Srikanth et al., 2015).

ZnO nanoparticles differ from each other in terms of size, shape, and crystal structure. These features depend on substrate type and its concentration, the kind of organic solvents used, applied temperature, and reaction time during synthesis (Altunbek et al., 2014). Zinc oxide is important due to its interesting characteristics such as chemical stability, high UV absorption, photostability, biocompatibility, and diversity of particles structures (Kolodziejczak-Radzimska and Jesionowski, 2014). It is used in different industries such as medical, pharmaceutical, cosmetics, textiles, and electronics (Mishra and Adelung, 2018). In recent years, inorganic compounds including metal oxides have received increased attention because of their high stability in harsh conditions and the fact that they are not harmful to humans, animals, and the environment (Stoimenov et al., 2002). Metal oxide nanoparticles (NPs) including ZnO have selective toxicity to bacteria while minimally affecting human cells. The reduced size of ZnO NPs increases their antibacterial characteristics (Seil and Webster, 2011).

Nanocomposite is a material consisting of two or more distinct phases where at least one of them is in nanoscale (between 1 to 100 nm). Nowadays, nanocomposites are

the subject of intense research owing to their unique mechanical, chemical, and physical properties (Camargo et al., 2009). Synthesizing polymeric nanocomposites using NPs can improve the optical and mechanical properties of the components of the nanocomposites; this improvement is because of the strong internal interaction between the organic polymer and the inorganic nanoparticles which have a large surface area and quantum effect (Sato et al., 2008; Safaei et al., 2019).

To our knowledge, no levan-ZnO nanocomposite has been synthesized to date; thus, its antibacterial effects have not yet been evaluated. The goal of this study was to optimize levan biopolymer production at lower costs, synthesize ZnO NPs, and manufacture levan-ZnO nanocomposite, as well as investigate its antibacterial effect on *Escherichia coli* and *Staphylococcus aureus* pathogens.

Material and methods

Levan polymer production

The bacterial *Zymomonas mobilis* PTCC 1718 was provided by the Iranian Research Organization for Science and Technology (IROST) and incubated at 30 °C for 48 h. The grown colonies were transferred to the inoculating culture media containing sucrose (50 g/l), yeast extract (7 g/l), K₂HPO₄ (2.5 g/l), (NH₄)₂SO₄ (1.6 g/l), MgSO₄·7H₂O (1 g/l), and incubated for 24 h at 28 °C without air and stirring. The Taguchi method and Qualitek-4 software were used to predict the best conditions for the production of levan. In this study, the effects of three factors (i.e., sucrose, yeast extract, and potassium phosphate) were investigated at three levels of levan synthesis (Table 1). The medium used for producing levan contained sucrose (150, 200, or 300 g/l), yeast extract (1, 1.5, or 2 g/l), K₂HPO₄ (0.5, 1, or 1.5 g/l), (NH₄)₂SO₄ (1 g/l), and MgSO₄·7H₂O (0.5 g/l). Following 20 min sterilization at 121 °C, the medium was inoculated with bacterial culture (2–5%) and kept in an incubator without shaking at 28 °C for 48 h. Then, the samples were centrifuged for 10 min at 5000 g. The supernatant was concentrated at 85–90 °C in a water bath in order to reduce the centrifuged volume to 1/3 (Silbir et al., 2014).

Levan polymer extraction

To extract the levan polymer, 90 ml of cold ethanol (96%) were added per 100 ml of concentrated solution. The samples were centrifuged at 4000 rpm following 2 h

Table 1. The Taguchi design of experiments and levan biopolymer production by *Zymomonas mobilis*

Experiment	Yeast extract [g/l]			KH ₂ PO ₄ [g/l]			Sucrose [g/l]			Levan [g/l]
	1	1.5	2	0.5	1	1.5	150	200	300	
1	1			0.5			150			21.66
2	1			1			200			19.00
3	1			1.5			300			57.00
4	1.5			0.5			200			34.33
5	1.5			1			300			26.33
6	1.5			1.5			150			8.16
7	2			0.5			300			41.33
8	2			1			150			11.60
9	2			1.5			200			15.83

of refrigeration. To determine the quantity of levan extracted, the sediments were put in an oven at 80 °C to be dried and then weighed (De Oliveira et al., 2007; Dos Santos et al., 2013).

Synthesis of ZnO nanoparticles

ZnO NPs were synthesized using the molten salt method. For this purpose, zinc chloride salt (83 g), sodium hydroxide (19.01 g) and potassium hydroxide (19.01 g) were combined, and placed in an oven at 220 °C for 45 min; the resulting mix was then cooled down to room temperature. Subsequently, the mix was rinsed in hot distilled water and centrifuged to remove alkali metal salts. The centrifuged sediment was placed in an oven at 100 °C for 2 h to remove moisture. Finally, a white color powder containing ZnO NPs was obtained (Sikalidas, 2011).

Levan-ZnO nanocomposite synthesis

ZnO NPs were homogenized in deionized water by a sonicator. Then, they were added to the biopolymer matrix in deionized water and placed on a stirrer for 1 h. In the next phase, the samples were transferred to a sonicator bath for 30 min. After oven-drying at 40 °C for 48 h, the nanocomposite powder containing levan polymer and ZnO NPs was obtained (Perez-Altamar and Perales-Perez, 2014).

Characterization

The UV-vis spectra were prepared for the synthesized levan biopolymer, ZnO NPs, and levan-ZnO nanocompo-

site in the range 200 to 800 nm by Agilent spectrophotometer. The FTIR spectra of the synthesized levan biopolymer, ZnO NPs, and levan-ZnO nanocomposite were prepared by an alpha spectrometer (Bruker, Germany). SEM images of the levan-ZnO nanocomposite were obtained by a TESCAN scanning electron microscope (Czech Republic).

Antibacterial activity

The antibacterial activity of the levan biopolymer, ZnO NPs, and levan-ZnO nanocomposite on *Staphylococcus aureus* from the Gram-positive bacteria and *Escherichia coli* from the Gram-negatives were examined by the disc diffusion method. For this purpose, a suspension of bacteria with an approximate concentration of 10⁸ CFU (colony forming units)/ml was prepared and cultured on a nutrient agar medium. Then, four discs containing the levan biopolymer, ZnO NPs, levan-ZnO nanocomposite, and Gentamycin (positive control) were placed on the plates and incubated for 24 h. All the tests were carried out with three experiment repetitions and three replicates of each experiment.

Results

Levan production

To determine the optimum conditions for levan production by *Zymomonas mobilis* PTCC 1718, the weight of the levan obtained from the nine separate experiments was measured (Table 1). The maximum production of microbial levan biopolymer, obtained in experi-

Table 2. The main effects of different levels of yeast extract, KH_2PO_4 and sucrose on levan biopolymer production by *Zymomonas mobilis*

Factors	Level 1 [value]	Level 2 [value]	Level 3 [value]
Yeast extract	32.53	22.94	22.92
KH_2PO_4	32.44	18.98	26.99
Sucrose	13.81	23.05	41.55

Table 3. Estimation of the effects of interacting factor pairs on levan biopolymer production by *Zymomonas mobilis*

Interacting factor pairs	Desirable levels	Severity index [%]
Yeast extract \times sucrose	(3.1)	29.51
Yeast extract \times KH_2PO_4	(3.1)	5.46
$\text{KH}_2\text{PO}_4 \times$ sucrose	(3.3)	5.39

ment 3, was 57 g/l. In this experiment, the most appropriate concentrations of the yeast extract, potassium phosphate, and carbon source to produce levan were 1 g/l, 0.5 g/l, and 300 g/l, respectively. The effects of different levels of the yeast extract (1, 1.5, and 2 g/l), KH_2PO_4 (0.5, 1, and 1.5 g/l), and sucrose (150, 200, 300 g/l) on levan production are presented in Table 2. Level 1 for yeast extract (32.53) and potassium phosphate (32.44), and level 3 for sucrose (41.55) had the best effect on levan production by *Zymomonas mobilis*. Estimating the effects of interacting factor pairs on levan biopolymer production by *Zymomonas mobilis* revealed that the interactions between the yeast extract and sucrose were the most relevant pair influencing the amount of levan produced (29.51%). On the other hand, potassium phosphate and sucrose had the lowest level of interaction (5.39%), which is very close to the interaction between the yeast extract and potassium phosphate (5.46%) (Table 3). Table 4 shows the analysis of variance (ANOVA) of parameters influencing levan production by *Zymomonas mobilis*. Yeast extract as a source of nitrogen and potassium phosphate had no significant effect on the amount of levan produced; sucrose as a carbon source was the main factor affecting the amount of levan (533.450). After evaluating the effect of the studied factors and their interactions, the best conditions for producing levan biopolymer were determined using the Ta-

guchi method (Table 5). The results showed that carbon source, yeast extract, and potassium phosphate, with a contribution of 15.41, 6.42, and 6.30%, respectively, resulted in the highest production of microbial levan biopolymer. Based on the prediction by the Taguchi method, level 3 for sucrose (300 g/l) and level 1 for yeast extract (1 g/l) and potassium phosphate (0.5 g/l) were presented as the most suitable levels for producing levan biopolymer.

Characteristics of levan-ZnO nanocomposite and its components

UV-vis spectrophotometry was used to study the optical properties of the synthesized levan biopolymer, ZnO NPs, and levan-ZnO nanocomposite. Absorption measurements were done in the range 250 to 600 nm. The observed changes in the peaks indicated that ZnO NPs were coated with levan biopolymers (Fig. 1).

In order to ensure that the production of levan biopolymer, the synthesis of ZnO NPs, and the formation of levan-ZnO nanocomposite occurred, FTIR spectrums were recorded for each sample (Fig. 2). In the range 900 to 1200 cm^{-1} , the observed peaks were due to C-C, C-O, C-O-H, and C-O-C bonds. The peak at 1063 cm^{-1} confirmed the production of levan. Furthermore, the peak at 435 cm^{-1} confirmed the presence of ZnO NPs. Considering the observed changes in the peaks of levan-ZnO nanocomposite compared to its components, the synthesis of nanocomposite was confirmed.

The SEM images of ZnO nanoparticles and levan-ZnO nanocomposite are presented in Figure 3. The SEM image of the nanocomposite shows that the ZnO nanoparticles were coated on the levan biopolymer and were desirably dispersed within the matrix.

Antibacterial analysis of the levan-ZnO nanocomposite and its components

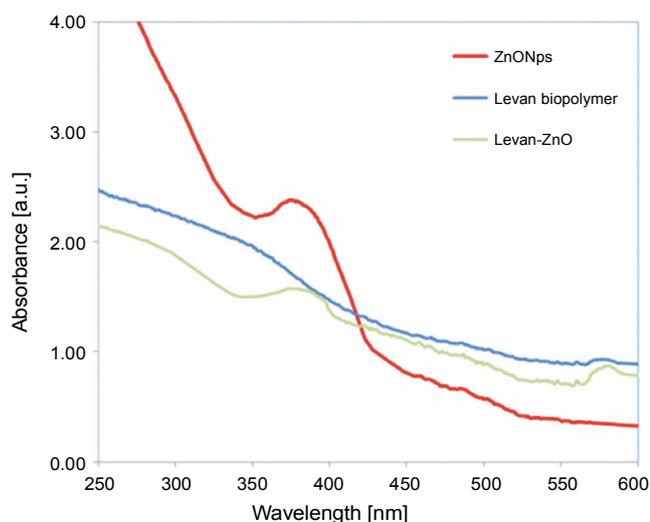
The antibacterial activity of levan biopolymer, ZnO NPs, and levan-ZnO nanocomposite against two pathogens (*Escherichia coli* and *Staphylococcus aureus*) were studied (Table 6). The inhibition zones of levan-ZnO nanocomposite: 16.00 mm and 14.66 mm for Gram-positive and Gram-negative bacteria, respectively, were significantly bigger than those of ZnO NPs (12.66 mm and 10.33 mm for Gram-positive and Gram-negative bacteria, respectively) and levan biopolymer (0 mm for both tested bacteria) ($P < 0.05$). No significant difference was

Table 4. Analysis of variance of the Taguchi experiment results for levan biopolymer production by *Zymomonas mobilis*

Factors	DOF	Sum of squares	Variance	Pure sum	Percent [%]
Yeast extract	2	185.22	92.61	0.00	0.00
KH ₂ PO ₄	2	275.21	137.61	0.00	0.00
Sucrose	2	1197.63	598.81	893.46	45.53

Table 5. Predicted optimum conditions for levan biopolymer production by *Zymomonas mobilis*

Factors	Level	Contribution [%]
Yeast extract	1	6.42
KH ₂ PO ₄	1	6.30
Sucrose	3	15.41
Total contribution from all factors		28.13
Current grand average of performance		26.14
Biopolymer production at optimum conditions		54.27

**Fig. 1.** UV-Vis spectroscopic analysis of ZnO nanoparticle, levan biopolymer, and levan-ZnO nanocomposite

observed in the antibacterial activity level of levan-ZnO nanocomposite and the antibiotic Gentamycin antibiotic (17.66 mm for Gram-positive and 15.33 mm for Gram-negative bacteria) against these pathogens ($P > 0.05$). The decrease in the growth of bacteria in the presence of the levan-ZnO nanocomposite in comparison with its components indicates its improved antibacterial ability in preventing the growth of both pathogens.

Discussion

As levan biopolymer is used in various fields, it is important to optimize its production. The results of this study show that the maximum level of levan production was obtained from *Zymomonas mobilis* PTCC 1718 in experiment 3 (57 g/l – Table 1). Levan culturing and production required the presence of three factors (carbon source, yeast extract, and potassium phosphate) in the growth medium; the tests revealed that sucrose had the greatest influence on levan production compared to the two other factors such that by increasing sucrose concentration, levan production also improved (Table 2). Compared to the previous studies on this subject, microbial levan production was at a higher concentration in this research (Melo et al., 2007). The authors studied the impact of four factors: temperature, agitation, sucrose, and yeast extract concentration on the production of levan by *Zymomonas mobilis*. The maximum amount of levan produced was 14.67 g/l, which was obtained at a temperature of 20 °C, agitation rate of 100 rpm, sucrose concentration of 250 g/l; the concentration of yeast extract was stated to have no effect (Melo et al., 2007). In another study, Silbir et al. (2014) studied levan biopolymer production by *Zymomonas mobilis* B-14023 in batch and continuous fermentation systems. In both

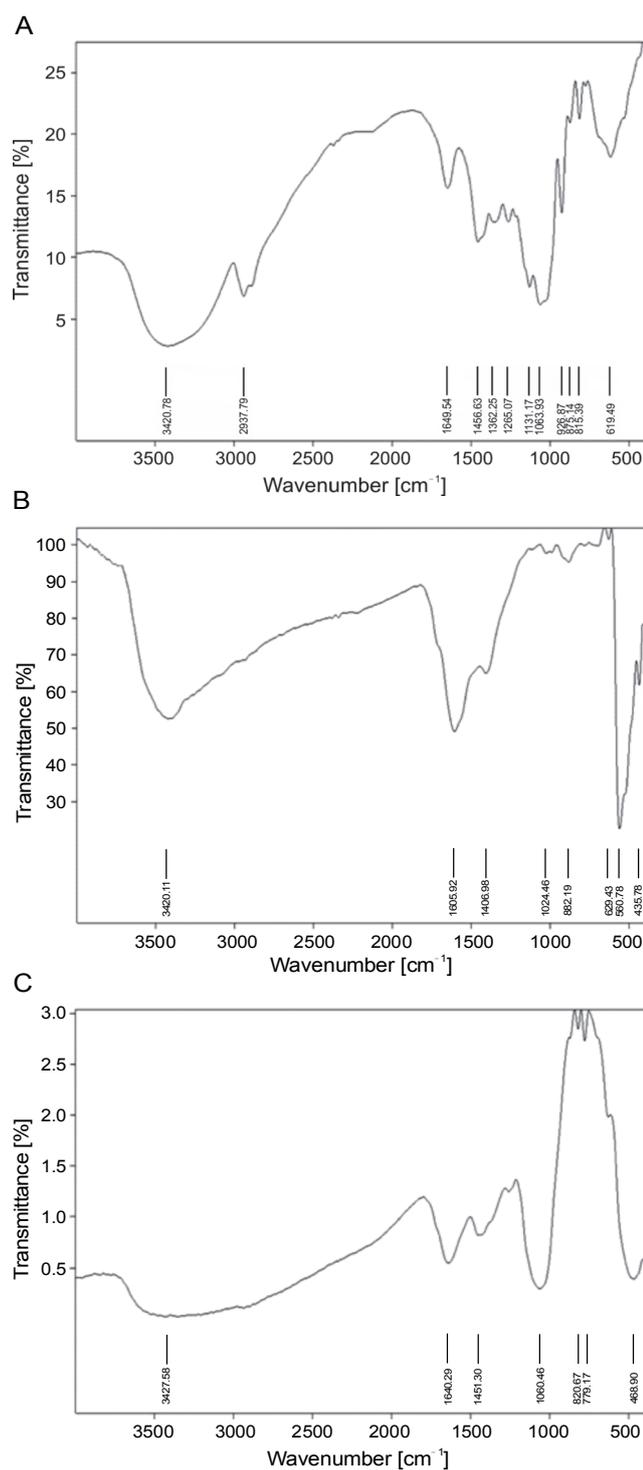


Fig. 2. A) FTIR analysis of levan biopolymer, B) ZnO nanoparticle, and C) levan-ZnO nanocomposite

systems, sucrose and numerous sources of nitrogen were used; yeast extract was found to be the best source of nitrogen. The authors noted that the maximum production of levan biopolymer in batch and continuous fermentation systems were 40.2 g/l and 31.8 g/l, res-

pectively. De Oliveira et al. (2007) used molasses, sucrose, and cane extract as a source of carbon to produce levan by *Zymomonas mobilis* ATCC 31821. The maximum production of levan biopolymer (21.685 g/l) was observed in the treatment containing sucrose while minimum production was observed in the presence of molasses (2.533 g/l) as the source of carbon. Moreover, earlier studies demonstrated that bacterial species and the amount of initial substrate play direct roles in producing levan biopolymer. Levan biopolymer production by *Bacillus subtilis* bacterium was reported to be 14.31 g/l by Shih et al. (2005); while Ghaly et al. (2007) reported that the amount of this biopolymer extraction from *Bacillus licheniformis* was 0.36 l/g. In a study conducted by Khanafari et al. (2010), the amount of extracted biopolymer from *Bacillus polymyxa* was 19.5 g/l.

In the present study, microbial levan biopolymer synthesis was optimized as a biodegraded polymer and then used for coating ZnO NPs in the synthesis of an antibacterial nanocomposite. By applying FTIR analysis and UV-vis spectroscopy, the synthesis of levan biopolymer, ZnO NPs, and levan-ZnO nanocomposite were confirmed (Fig. 1 and Fig. 2). The desirable antibacterial properties of the synthesized levan-ZnO nanocomposite make it suitable for medical, environmental, food industry, and pharmaceutical applications.

An important application of the synthesized nanocomposite, taking into consideration the antibacterial properties of levan biopolymer and ZnO, is its use as a new antibiotic. Since pathogens have become increasingly resistant to available antibiotics, finding a new biocompatible antibiotic is of great importance. While studying the antibacterial activity of levan-ZnO nanocomposite against two pathogens of *Escherichia coli* and *Staphylococcus aureus*, it was observed that the nanocomposite had the ability to prevent the growth of both pathogens (Table 6). Recent developments in the field of nanotechnology, especially in the making of nanoparticles of different shapes and sizes, have led to the establishment of a new group of antibacterial agents (Helminger et al., 2016; Taran et al., 2016). Nanoparticles have a higher surface per volume ratio compared to larger particles with the same chemical composition, which makes them more active biologically (Rad et al., 2018). Nanocomposites are nanostructures that have been regarded and studied as a new class of antibiotics in recent years due to their unique features. Droval et al. (2008)

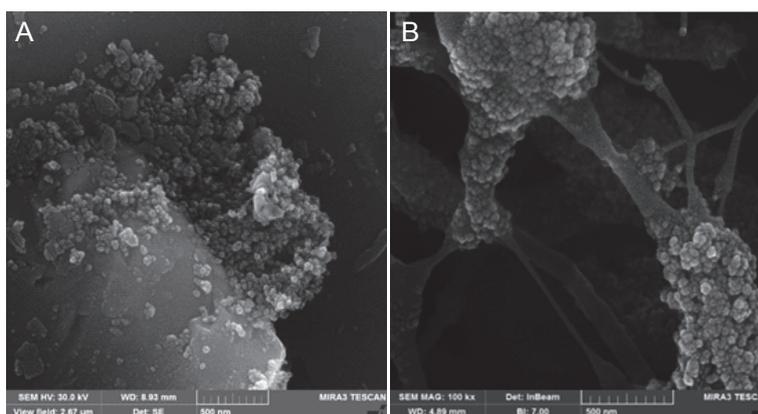


Fig. 3. A) SEM analysis of ZnO nanoparticles and B) levan-ZnO nanocomposite; the image of the nanocomposite shows that the nanoparticles are coated on the biopolymer

Table 6. Comparison of antibacterial activity of levan-ZnO nanocomposite with ZnO nanoparticles and levan polymer

Factors	Gram-positive bacteria (<i>Staphylococcus aureus</i>)	Gram-negative bacteria (<i>Escherichia coli</i>)
	zone of inhibition [mm]	zone of inhibition [mm]
ZnO	12.66 ^b	10.33 ^b
Levan	0.00 ^c	0.00 ^c
Levan-ZnO	16.00 ^a	14.66 ^a
Gentamycin	17.66 ^a	15.33 ^a
Standard error of the mean	0.39	0.31

synthesized nanocomposites using two polymers of polyamide 6) PA6 (and low-density polyethylene) LDPE (as a matrix of ZnO NPs and examined their antimicrobial properties against *Escherichia coli* and *Staphylococcus aureus* pathogens. The authors found that PA6-zinc oxide nanocomposite had better antibacterial properties compared to pure ZnO particles (Droval et al., 2008). In another study, Hadory and Shim (2013) synthesized a hybrid compound of ZnO and chitosan using a precipitation method. The authors examined the antibacterial activity of this nanocomposite against *E. coli* and observed that it had the ability to eradicate this bacterium (Haldorai and Shim, 2013). Perez-Altamar and Perales-Perez (2014) successfully synthesized chitosan/cellulose-ZnO nanocomposite; they examined its antibacterial activity and reported the ability of this nanocomposite to prevent the growth of *E. coli* pathogen. Dhillon et al. (2014) synthesized ZnO-chitosan nanocomposite using

two methods of spray drying and depositing. The antibacterial effects of the synthesized nanocomposite were examined against the pathogens of *Micrococcus luteus*, *Candida albicans*, and *Staphylococcus aureus*. The maximum antimicrobial effect against *M. luteus* and *S. aureus* was observed at concentrations of 0.156 to 0.625 mg/ml of the nanocomposite (Dhillon et al., 2014).

The results of the present study and the previous ones described here reveal that the formation of nanocomposites composed of ZnO nanoparticles and a polymer improves their antimicrobial properties. Considering their tendency to agglomerate, the use of biopolymers as biodegradable stabilizers can prevent this agglomeration and increase effective surface area against pathogens. To our knowledge, no study has reported the synthesis of a levan-ZnO nanocomposite yet. Therefore, the nanocomposite was synthesized and tested as a new antibacterial substance in this study. This novel nanocompo-

site with antibacterial properties has a great potential for use in various fields, for example, in medical, food, pharmaceutical, dentistry, and other industries.

Conclusions

Levan was produced in optimum conditions by *Zymomonas mobilis* PTCC 1718. In optimum conditions, the amount of extracted biopolymer was 57 g/l; this study showed that there is a direct relation between sucrose concentration and the production of levan biopolymer. The production of levan biopolymer, the synthesis of nanoparticles, and the formation of nanocomposite were all confirmed by UV, FTIR and SEM analysis. The results of an antibacterial test showed the ability of levan-ZnO nanocomposite in reducing the growth of pathogenic microorganisms of *Escherichia coli* and *Staphylococcus aureus*. Based on the results of this study, it seems profitable to use levan-ZnO nanocomposite as a practical antibacterial substance in various industries.

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