

Production of New Nano-Bacterial Cellulose with *Lactobacillus rhamnosus* by Using Whey Waste as Substrate with Optimization Taguchi Method, which has the potential to be used in many biomedical products

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Abstract

Whey waste, which has a negative impact on the environment, is an important component with high organic content. The fact that it contains lactose, a fermentable sugar, is a suitable substrate for the formation of natural nano-cellulose. Bacterial nano-cellulose (BNC), a type of natural cellulose polymer synthesized by some microorganisms, has been reported to be a promising natural biomedical material due to its distinctive feature, including its unique fibril nanostructure, high water holding capacity, crystallinity, high chemical purity, fine wet mechanical property.

In this study, new BNC production was realized for the first time by using *Lactobacillus rhamnosus* bacteria and whey as organic substrate. Optimum condition was determined by Taguchi method under the following condition; pH (5-6), organic source concentration (25-100 % g/L), active culture (10-30 % g/L), incubation period (8-12 day). Whereas Taguchi method was highest performed at at pH 5.5, organic source concentration 25 % g/L, active culture 30 % g/L, incubation period 8 days with 5.41 g BNC yield. Effects of organic source concentration found as decisive factor on *Lactobacillus rhamnosus* BNC yield with 95% confidence interval. Field emission scanning electron microscopy (FESEM), fourier transform infrared spectroscopy (FTIR), differential / thermogravimetric thermal analysis (DTG/TG) were utilised to evaluate the structure and characterization of BNC.

BNC production by *Lactobacillus rhamnosus*, with its biocompatible and biodegradable properties, environmentally friendly and low-cost nanomaterials have been produced with the potential to be used in many biomedical applications such as wound dressing and drug coating material.

1. Introduction

Bacterial cellulose (BNC) known as a nanomaterial and could be produced by some bacteria such as *Gluconacetobacter*, *Acetobacter* spp., *Rhizobium* spp., *Agrobacterium* spp., *Alcaligenes* spp., *Achromobacter* and *Sarcina* [1, 2, 3, 4]. The BNC biosynthesis is a regulated process including enzymes, catalytic complexes reactions and can be investigated with two steps such as intracellular production of 1,4-b-glucan chains and crystallization of cellulose chains [3].

BNC is native cellulose which has high crystallinity, water capacity and strength also biocompatible with plant cellulose [4]. Also it produces much thinner 3D dimensional nanoporous networks with approximately 30–50 nm than plant cellulose [5]. It has the same molecular formula as BNC and plant celluloses $(C_6H_{10}O_5)_n$ but BNC doesn't contain lignin, pectin, and hemicelluloses; thus BNC purification doesn't require harsh chemicals and also purification is easy to apply [6]. Having high surface area and high porosity, BNC is a material that shows high performance with other compounds in physical interaction [3, 7].

This article presents an alternative strategy for the future using whey, which is waste, as well as the role of raw material for low-cost production of bacterial cellulose. Whey obtained from whey as a by-product of the dairy industry is considered a pollutant due to its high biological oxygen demand and high cost of

evaluation [8]. Besides, it has rich nutrient content [9]. Lactic acid bacteria are generally used in dairy products, as pure culture. LABs are organisms that can be isolated from natural environments such as most people, animals and plants, adapted to a specific environment and are found in almost all natural habitats. *Lactobacillus rhamnosus* strains with probiotic properties have begun to be used in new types of yoghurt [10]. With the increasing demand of consumers for probiotic products, the production of lactic acid bacteria in the industrial sector has gained importance [11].

In addition to the effects of other factors in obtaining bacterial cellulose, it is crucial to determine the optimum values of some factors such as fermentation time and pH that maximize the bacterial cellulose yield [12]. In the present study, bacterial nano-cellulose (BNC) production was optimized based on different concentration substrate, as well as temperature, pH, and incubation time. According to the objectives of this study were determined as identifying whether the bacteria is producing *Lactobacillus rhamnosus* BNC for the first time in literature, identify an optimal media formulation for improved BNC production and characterization BNC production using fourier transform infrared spectroscopy (FT-IR) and field emission-scanning electron microscopy (FE-SEM). The substrate and process variables needed are optimized to ensure maximum BNC production at low costs. In optimization studies, while all other parameters are kept constant, one parameter is varied. In this case, it is not possible to evaluate the interaction of the parameters with each other. For this reason, Taguchi's method was used in this study to determine the effects of the factors affecting the BNC production process. Also, the evaluation of lactose in whey, which is considered as waste water with a complex organic matter content, it provides the advantage of environmentally friendly application to this study.

2. Materials and Methods

2.1. Procurement of whey

Cheese whey was used as organic source and kindly obtained from Isparta Ünsüt Company (Turkey). Under aseptic conditions, samples were collected and stored at 4°C in the laboratory. Then samples were stored at -20°C before the analysis. It contains 60–62% lactose. The pH of the whey was adjusted to 6. Protein precipitation was induced by autoclaving from the whey at 121 °C for 20 min and after removed by filtration.

2.2. Microorganism and Culture Media

Lyophilized culture of *Lactobacillus rhamnosus* (DSM, DELVO-ADD 100R, Netherlands) was used in all experiments. The stock culture of bacterial strain *Lactobacillus rhamnosus* was stored at -80°C in MRS medium as 15% glycerol. Precultures were per liter prepared in the 100 mL MRS culture medium containing, 1g peptone, 2g glucose, 1g beef extract, 0.2g ammonium citrate, 0.01 g magnesium sulphate, 0.5g sodium acetate, 0.005 g manganese sulphate, and 0.2 g di-potassium phosphate. A 5 mL of this culture was used for further inoculation in the 100 mL MRS production media. The media contains molasses, yeast extract, MnSO₄.4H₂O, Tween 80, urea, and CaCO₃. Table 1 shows the design of the experiments on the set of orthogonal arrays with respect to the selected factors and levels designed with

using the taguchi method. The effect of organic sources concentration, active culture, pH and incubation time on the production of bacterial cellulose was investigated using the bacteria *Lactobacillus rhamnasus*.

Table 1
Design of experimental factors and levels

Factors	Level 1	Level 2	Level 3
pH	5.0	5.5	6.0
Organic source concentration	50% Whey	25% Whey	75% Whey
Amount of active culture	10%	20%	30%
Incubation time (day)	8	10	12

2.3. Production of BNC

Fermentations were performed as batch fermentations with *Lactobacillus rhamnasus*. It was cultivated in 100 mL liquid medium in 250 mL erlenmeyer flasks. Neutralizing agents were added into fermentation medium to control the pH during the fermentation. Calcium carbonate was used as neutralizing agent due to using widely for flask investigations and bioreactor processes [13]. Also, in this study, CaCO₃ (60% (w/v) of the initial lactose concentration) was used as a neutralizing agent. The medium was inoculated with about (10% w/v) inoculum of bacteria. Fermentations were carried out with a temperature controlled orbital shaker at a stirring speed of 150 rpm and fermentation temperatures were chosen as the optimum growth temperatures given in Table 1. All experiments were carried out in duplicate.

2.4. Purification of BNC pellicles

After BNC was produced at 30 oC for 8 days in optimum conditions, the BNC pellicles were harvested by discarding the medium and washing with large amounts of nanopure water. The pellicles were then rinsed three times with 0.1M NaOH (Sigma Aldrich, Germany) at 60°C for 4 hours to remove sticky bacteria. Finally, the samples were repeatedly washed until the pH was below 7.0 with copious amounts of distilled water. Until a constant weight was reached the wet cellulose pellets were dried at 60°C, then the concentration was determined as g / L (mass (g) BNC / volume of culture medium (L)). For further instrumental analysis isolated pellicles were used.

2.5. Characterizasyon of BNC

The The microbial cellulose obtained as a result of incubation was ground in a laboratory mill (Retsch GE) after washing and drying, and particle size was obtained as 40 mesh. Viscometer measurements were performed in cuvettes with 35 ml chambers. 3.5 g of cellulose was added and the volume of the solution was completed to 35 ml with distilled water. The viscosimetric values of this 10% suspension (Vibro Viscometer SU-10) were measured in mPa.s by taking a reading every 20 seconds in the 20–21°C range, 30 Hertz frequency, constant vibration.

The shaking method was used in order to measure the water holding capacity of the samples [14]. Two shakes were made and weighed to remove excess water from the surface of the samples. The samples were dried at the room temperature for 48 hours and then dried at 60°C for 12 hours to completely remove the water content. The water holding capacity was calculated using the following method:

$$\text{WaterHoldingCapacity} = \frac{\text{Massofwaterremovedduringdrying (g)}}{\text{Dryweightofbacterialnano - cellulose (g)}}$$

1

Thermal gravimetric analysis was performed to measure the weight loss and BNC thermal stability of the analyzed material when the temperature increased linearly [15]. Thermal degradation behavior of bacterial cellulose was investigated at 0-900 oC using the TGA / TDA device (Perkin Elmer diamond, USA). Percent and derivative weight loss were recorded against temperature for all samples.

Solubility characteristics of microbial cellulose were determined using various solvents. The solvents used for solubility tests are toluene, acetone, chloroform, benzene, carbon tetrachloride, and dimethylformamide. In the experiment, 0.1 g of sample was placed in 10 mL of solvent and the tubes were rinsed at room temperature for 24 h to determine their solubility in solvents [16].

To determine the elemental contents of the BNC samples obtained as a result of experimental studies, FT-IR Spectroscopy was evaluated. It has been concluded whether pure cellulose is obtained from the numerical percentage of the elemental C content in the FT-IR bands of the cellulose sample. FT-IR analysis was carried out in Süleyman Demirel University Experimental and Observational Student Research and Application Center Laboratory. FT-IR spectra of the BNC pellicle were obtained with Perkin-Elmer FT-IR spectrophotometer (Norwalk, USA) in diffuse reflectance mode at a resolution of 4 particles/cm in KBr pellets.

Fe-SEM was used to determine the surface structure of BNC cellulose samples obtained after drying for 10 days at 28°C in the liquid medium. Fe-SEM analysis was carried out at Isparta Süleyman Demirel University Energy Research and Research Application Center. Microstructures and morphology of samples were evaluated by examining images in three dimensions with TEM..

2.6. Taguchi Methodology

Taguchi is one of the impressive ways to design an experiment to optimize the process. Unlike traditional full factor research, it can be used as a powerful tool to optimize performance characteristics of process parameters. The Taguchi method uses orthogonal array design (OAD) while designing less number of experiments with a large number of parameters [17]. The signal / noise (S / N) ratio enables the quality characteristics of the process to be measured precisely at different process levels of controllable and uncontrollable factors. In addition, it makes it possible to minimize the negative effects of uncontrollable factors on the process. It is possible to provide the optimum parameters of each parameter in the process by determining the S / N ratios.

The measurement of the quality of the results is measured by the signal-to-noise (S / N) ratio with three different characteristics of the target values: "larger is better", "smaller is better" or "nominal is better" [18]. In this study, target values of "smaller is better" was chosen. Because it is aimed to determine the highest production at the lowest concentrations of the parameters. Thus, it is determined how the effects of the selected parameters at the lowest levels affect the BNC production efficiency. In this study, interactions between factors were not taken into account and the main effects of four parameters affecting the process were evaluated.

2.7. Design of Experiments and Choice of Factors

In this study the Taguchi method was used as an experimental design and analysis method. The L9 orthogonal arrays improved by Taguchi method. The parameters determined by examining the literature information and their levels are given in Table 2. BNC production was determined by optimization conditions, four different variables were selected as pH, carbon sources concentrations, amount of active culture conditions, and incubation time. Also, 3 different levels were determined for each parameter. The main steps of this method was created firstly determination of factors, interactions and the levels of each factor. Minitab 16 software (Minitab Inc., USA) was used to create the experimental design and evaluate the data. To determine the optimization of the BNC manufacturing process, how various factors influence the process individually as well as how they contribute to the complexity of the entire process and their multiple interactions were also evaluated. By measuring the predicted interactions of different factors (based on the severity index (SI)), the effects of four different factors used in the experiment set at various levels were determined. Data obtained from Taguchi design experiment and all parameters were subjected to analysis of variance (ANOVA) for the significant difference between BNC production efficiency. P values less than 0.05 were considered significant. All statistical analyzes were performed using MINITAB v.16 (Minitab Inc., USA) statistical software package.

Table 2
Taguchi L9 orthogonal experimental design

Experiment No	pH	Organic source concentration (%)	Active culture (%)	Incubation time (day)
1	5.0	25	10	8
2	5.0	50	20	10
3	5.0	75	30	12
4	5.5	25	20	12
5	5.5	50	30	8
6	5.5	75	10	10
7	6.0	25	30	10
8	6.0	50	10	12
9	6.0	75	20	8

3. Results and discussion

3.1. Taguchi Method

In this study, three separate levels were defined for each of the BNC production process parameters and 81 separate experiments were required to complete the process. Instead, experiments were carried out by applying the L9 orthogonal array scheme, which requires nine experiments with the taguchi method. Experimental results were evaluated according to the Taguchi OA L9 method. Figure 1. shows main effect plots for S/N ratio for BNC production in *Lactobacillus rhamnasus* with different variables, including pH (5–6), organic source concentration (25–100), active culture (10–30), incubation period (8–12). The main effect graphs showing the signal-to-noise (S / N) ratio describing the scattering around a target value were obtained by using Taguchi method. Noise factors are seen as the reason for the variability of the answers examined for the target value. S / N ratio measures the performance level and the effects of noise factors on performance. The variance decomposition is obtained for the relative effects of different production parameters on BNC productivity yield, which is called variance analysis (ANOVA) [19]. The ANOVA was investigated to find the effects of process parameters on BNC production. Different levels of BNC production were evaluated the effect of individual parameters for the conversion. According to Taguchi Method, the optimum *Lactobacillus rhamnasus* production conditions was obtained at pH 5.5, organic source concentration 25%, active culture 30%, incubation period 8 days.

In the case of the design with the Taguchi method, when the experimental data and the estimation data were examined, 4.3 g BNC yield was obtained at pH 5.8 and the organic source concentration was 40%. While, the active culture amount was 10% at pH 5.5, 5 g BNC yield was obtained. If the organic source

concentration was 70% and the active culture amount was 25%, the amount of BNC yield was 2 g. When the organic source concentration was 70% and the incubation time selected as 10 days, the amount of BNC yield was 2 g. 0.2 g BNC yield was obtained with 20% amount of active culture and 10 days incubation time.

The Taguchi method based on OA detects optimum levels of process parameters by reducing variance for experiments. The results were then converted into S / N ratio data. S / N ratio of Taguchi method was used for data analysis and estimation of optimum parameters. Taguchi method factor design gives good results in the effect studies of various combinations. Figure 2. shows the effects of four parameters such as pH, organic source concentration, incubation time and active culture concentration on BNC yield using Taguchi experimental design. pH and incubation times have been shown to positive effect on production efficiency by affecting BNC production. The effect of organic source concentration varies depending on the amount of active culture. Increasing in the amount of active culture and increasing the number of individuals in the medium caused a decrease in BNC efficiency due to the insufficient organic source in the medium. The relative interactions of the factors on the BNC process performance are shown in Table 3. Table 3 shows that tested model has shown that model effectiveness was found as $R^2 = 96.22\%$. Analysis of variance (ANOVA) determined the significance of each independent variable which was presented in (Table 4). With 95% confidence interval, all parameters have marginally significant effects ($P > 0.05$).

Table 3
General Linear Model: BNC value versus pH; organic source, active culture analysis of variance for BNC value, using sequential SS for tests.

Source	DF	Seq SS	Adj SS	Seq MS	F	P
pH	2	4.6769	4.6769	2.3384	2.49	0.409
Organic source concentration	2	9.1785	9.1785	4.5892	4.89	0.305
Active culture	2	4.4193	4.4193	2.2096	2.35	0.419
Incubation period	1	5.5873	5.5873	5.5873	5.95	0.248
Error	1	0.9384	0.9384	0.9384		
Total	8	24.8004				
S = 0.968736 R-Sq = 96.22% R-Sq(adj) = 69.73%						

Table 4
Analysis of variance (ANOVA) evaluation on BNC yield.

Experiment No	pH	Organic Source Concentration (g/L%)	Active Culture (g/L%)	Incubation time (day)	BNC value (g)	SNRA1
1	5.0	25	10	8	0.06	24.4370
2	5.0	50	20	10	1.13	-1.0616
3	5.0	75	30	12	0.67	3.4785
4	5.5	25	20	12	0.00	*
5	5.5	50	30	8	0.23	12.7654
6	5.5	75	10	10	0.00	*
7	6.0	25	30	10	0.00	*
8	6.0	50	10	12	5.41	-14.6639
9	6.0	75	20	8	0.00	*

3.2. FTIR

Figure 3 indicates FTIR spectra of *L. rhamnosus* cellulose. According to the analysis results, the broad band intramolecular and intermolecular hydrogen bonding -OH stretching at 3430 cm⁻¹ observed in the FTIR spectrum of the BNC sample obtained from *L. rhamnosus*.

Other researchers also found major stretching peak associated with -OH groups on the glucose rings and water molecules take place between 3300 cm⁻¹ and 3600 cm⁻¹ [20, 21]. Ali et al., (2001) found that the C-H stretching vibration bands of -CH₃ and -CH₂ groups at 2926 cm⁻¹ whereas bending bands of these groups at 1458 and 1562 cm⁻¹. Also, in our study the intensities of bands at 2926 cm⁻¹ and 1628 cm⁻¹ were observed for *L. rhamnosus* cellulose [22].

FTIR results showed that intensities of bands at 2926 cm⁻¹ and 1628 cm⁻¹ were monitored for *L. rhamnosus* cellulose. This peak intensity is indicative of the existence of more aliphatic carbon groups (-CH₂-). These spectra of standard and produced cellulose showed that these compounds obtained from different media. The band at 1628 cm⁻¹ is associated with water in cellulose and probably some hemicelluloses [23, 24, 25]. In a study with microcrystalline cellulose and bamboo fibers, the H-bound OH groups were seen at 3340–3412 cm⁻¹ wave length, and the broader band gap CH tension was similar to our study (2968 – 2900 cm⁻¹) [26].

3.3. Water Release Feature

The BNC yield was found as 60.4 g/g for *L. rhamnosus* bacterium. Water release rate of cellulose produced by *L. rhamnosus* bacteria was determined as 64.4%. The water release rate of celluloses during

the drying period was determined as 28.4%. The water release process of the cellulose produced by the *L. rhamnosus* bacterium at the end of the 7 hours. The WHC of the BNCs produced in this study was in a suitable agreement with literature-cited publications [27].

The viscosity value of *L. rhamnosus* BS solution was found as 36 mPa.s at 0. Seconds (Fig. 4.). A linear decrease in viscosity values was observed by dilution of BS solution. It was determined that the viscosity decreased from 36 mPa.s to 31.2 mPa.s within 3 minute.

The WHC (water holding capacity) was considered as an most important physical characteristics due to biomedical application of BNC. The variations between the WHC were related with porosity and surface area. The greater surface area and larger pore size allow more water to penetrate and indwell in the BNC matrix.

3.4. Thermal properties

Figure 5. shows the thermogravimetric /differential thermal analysis (TG/DTG) curves of *L. rhamnosus* BNC sample. The thermal degradation behaviors of cellulose produced by *L. rhamnosus* were investigated and the temperatures of decomposition were determined. The graphic in Fig. 5 shows that thermal degradation occurs in two different stages.

Mangut et al. (2006) found that first degradation step was started at 250°C and ended about 430°C and peak was obtained at 350°C. It means that it is cellulosic materials. At this temperature, simple structures such as CO, CO₂, H₂O, CH₄, CH₃OH are being removed by decarboxylation and demethoxylation. The second decay was observed at 780°C [28]. In this step, the aromatic components are probably separated more slowly. Due to the pyrolysis and decomposition of the cellulose concentration it is seen that a slower weight loss from 350°C to 700°C to form a crosslinking ring structure. Many studies have shown the behavior of cellulose in inorganic and organic solvents. Although the ability to dissolve in various solvents is of importance for the usage of BNC in different industry, it is also important to reveal the effects of organism differences on the cellulosic composition. Toluene, acetone, chloroform, benzene, carbon tetrachloride and dimethylformamide solvents were used for solubility tests of the cellulose samples produced by *L. rhamnosus* bacterium.

In the experiment, 0.1 g sample was placed in 10 ml of solvent and the tubes were shaken for 24 hours at room temperature, and their dissolution ability in the solvents was determined. As a result of the solubility test, it was determined that BNC was dissolved only in toluene solvent in 80°C water bath. Solubility, breaking hydrogen bonds between polymer chains and increasing the accessibility to the intercrystalline structure can be explained.

The DTG curve for *L. rhamnosus* BNC sample showed that higher temperatures was shifted by increasing crystallite size. This behavior suggests that higher crystallite size shows higher thermal stability. Kim et al. (2010) also found that an increase in the crystallite size promoted by higher thermal stability. Therefore, BNC sample has probably higher thermal stability because it has higher amount of hydrogen

bonds between cellulose chains and leads to more ordered and packed cellulose regions [29]. High crystallite BNC can possibly turn into a increasing of the thermal decomposition temperature of cellulose. The intensity of L. Rhamnosus BNC, which is believed to be rich in aromatic structures, supports the FTIR analysis results.

Figure 6. shows the cellulosic structure produced by FE-SEM electron microscopy images of L. rhamnosus bacteria with x400 and x24000 magnification, respectively. Figure 6. shows that it was determined that the cellulosic fibers formed a tubular network structure. The cellulose fibers in these images were reinforced by determining their chemical and physical properties.

4. Conclusion

In this study, the production of BNC was optimized using different variables such as pH, organic source concentration, active culture concentration, and incubation period by Taguchi method. Characterization tests showed that the produced BNC revealed a strong network of nano fibrils. In addition, it does not involve toxic and any kind of hazardous materials in producing BNC, which is excellent and suitable for safe environments such as medical, a packing material in fields such as wound healing, drug coating, vein production, food industry and cosmetics applications. In conclusion, BNC produced from L. rhamnosus could play an crucial and environmentally friendly role in diverse medical and industrial applications as another way to cellulose obtained from other sources, such as microorganism and plants.

Declarations

Ethical approval is not required. There is no funding received. The dataset was not used.

Author Contribution

A.B. and C.D. wrote the body of the manuscript and all authors reviewed the manuscript.

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Figures

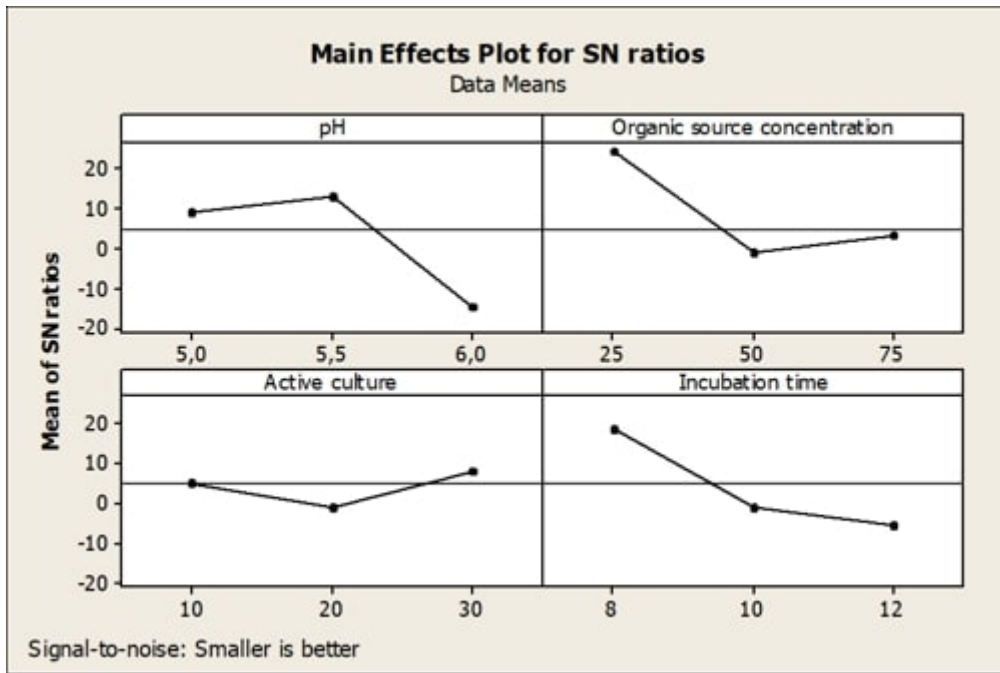


Figure 1

Main effect plots for S/N ratio between BNC production in *Lactobacillus rhamnasus* with different variables. — Parcelles à effet principal pour le rapport S/N entre la production de BNC dans *Lactobacillus rhamnasus* avec différentes variables.

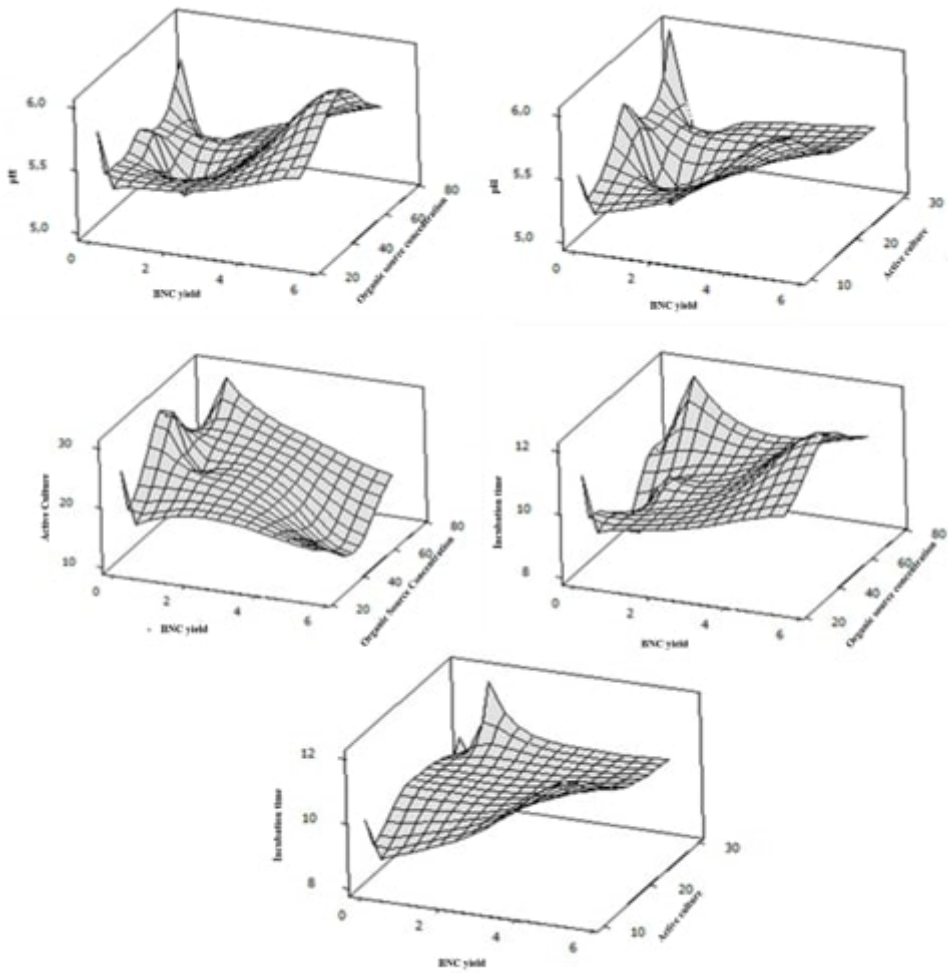


Figure 2

Effects of the parameter on the BNC production. – Effets du paramètre sur la production de BNC

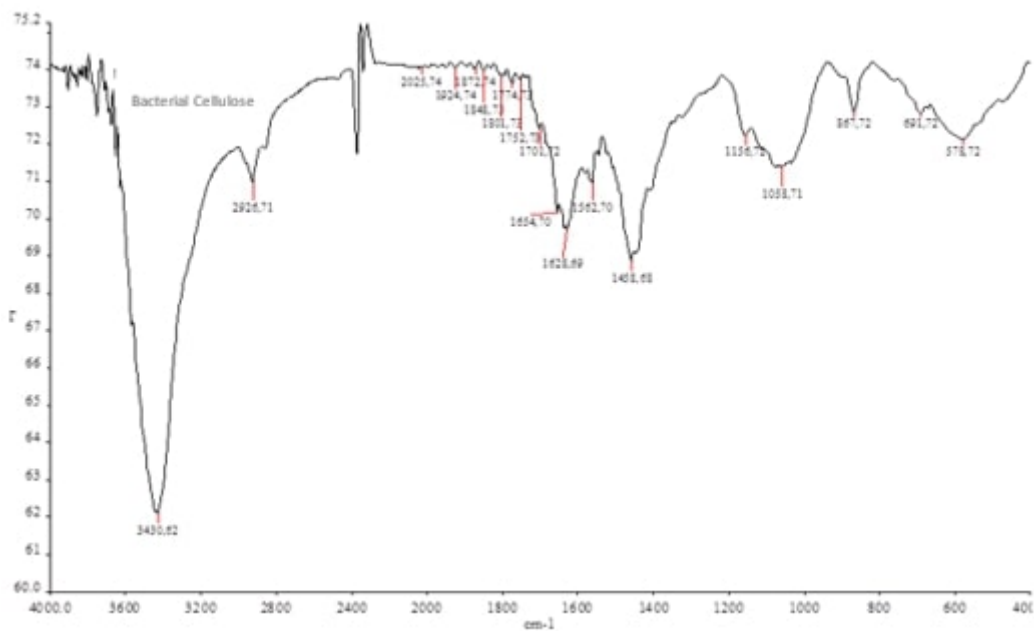


Figure 3

Cellulose FTIR diagram produced by *L. rhamnosus* bacteria. —Diagramme FTIR de cellulose produit par la bactérie *L. rhamnosus*

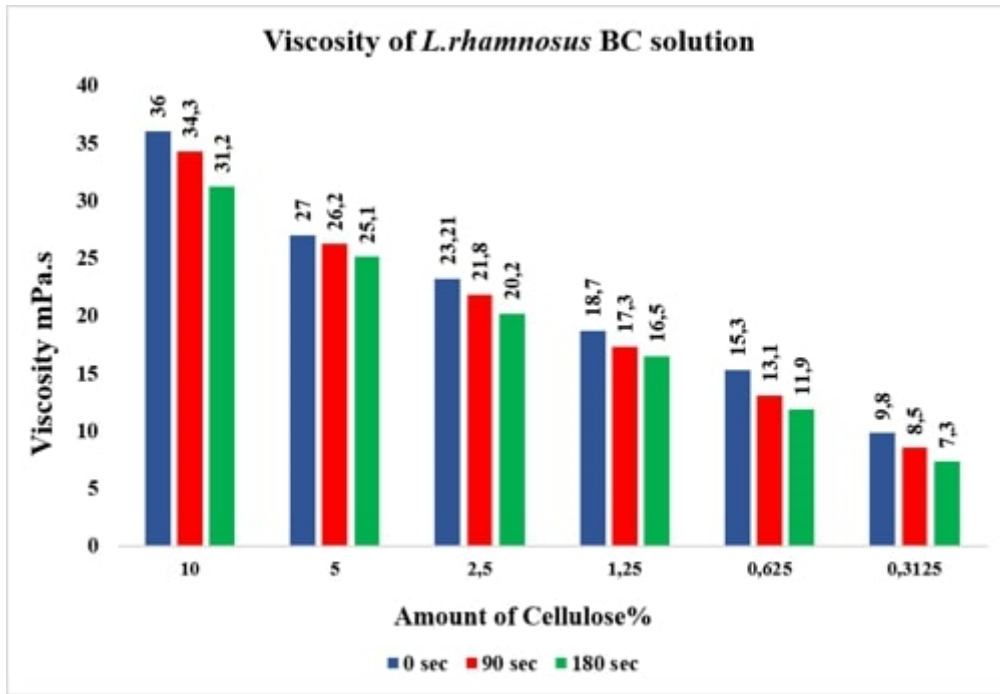


Figure 4

Viscosity of *L. rhamnosus* BNC solution. — Viscosité de la solution *L. rhamnosus*BNC.

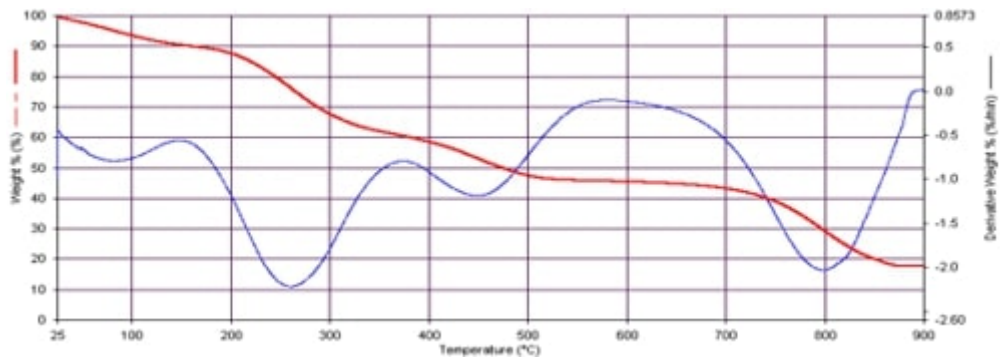


Figure 5

Cellulose TGA and DTG chart obtained from *L. Rhamnosus*. — Graphique cellulose TGA et DTG obtenu après de *L. Rhamnosus*

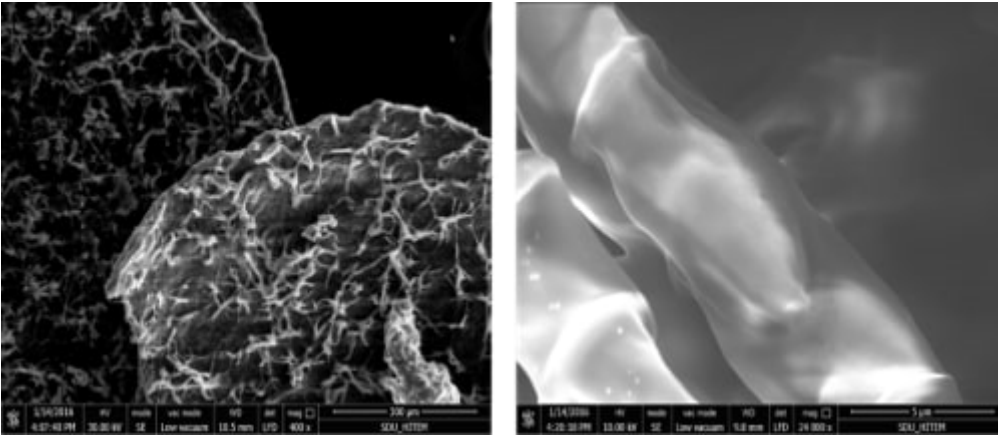


Figure 6

FE-SEM electron microscopy images of *L. rhamnosus* bacteria. – Images de microscopie électronique FE-SEM de la bactérie *L. rhamnosus*