IMPROVEMENT OF BACTERIAL CELLULOSE PRODUCTION BY *ACEOBACTER XYILINUM* DSMZ-2004 ON POOR QUALITY HORTICULTURAL SUBSTRATES USING THE TAGUCHI METHOD FOR MEDIA OPTIMIZATION. PART I

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The present paper investigates bacterial cellulose (BC) biosynthesis by *Acetobacter xylinum* DSMZ-2004, a source of microbial exploitation for cellulose obtainment, using poor quality apple extract and glycerol as carbon source.
The objective of this research was to determine the influence of some significant biosynthesis medium parameters on bacterial cellulose production by enhancing the value of inadequate quality apples in a substrate combined with glycerol, leading to their superior conversion. Significant cost reduction is possible, along with an improvement in production efficiency and profitability, the lower limit of microbial cellulose cost being determined by the price of the raw matter used as a substrate.
Inadequate quality apples and glycerol as a by-product of biodiesel production are not only cheap, but also abundant. It has been proven that bacterial cellulose production has been improved by applying the Taguchi experimental model of culture medium optimization, the maximum BC yield being of 8.6 g/L.

Keywords: bacterial cellulose, poor quality apples, medium optimization, Taguchi method

INTRODUCTION

Most of the time, fruits are sold and consumed in their natural state, but most of the ones that are deteriorated or do not correspond to size standards, are processed into jams and sauces.

Bad weather and other natural disasters lead to fruits presenting inadequate quality and, implicitly, to low prices and fruit waste. The great majority of these fruits end up being thrown away.

Apples are popular fruits cultivated in our country, available most of the year. Considering their rich content of carbohydrates, proteins and oligoelements, we have in view to use such fruit of poor quality as a natural substrate for useful products fermentation.

In comparison with other natural plant cellulose sources, bacterial cellulose (BC) is one of the most promising basic biological material, which presents many unique properties, including high purity and crystallization, high polymerization degree, water absorption and retention capacity, high resistance to stretching and strong biological adaptability.[1-6]

This type of material with wide application perspectives brings extraordinary economic benefits in various fields, such as food, textiles, paper, composite membranes, medicine, artificial skin and blood vessels, binders, diaphragms, speakers.[7-14]

In recent years, the predominant applications of bacterial cellulose have been in the biomedical field, being extremely useful for wound dressings, artificial skin, dental implants, vascular grafts, covering catheter, membrane dialysis, coating for cardiovascular and cranial stent, membranes for guided tissue regeneration, tissue replacement, drug carrier for controlled release, vascular prosthetic devices, tissue engineering matrix and artificial blood vessels.[15]

Microbial cellulose proved to be a remarkably versatile biomaterial and if this biopolymer could be successfully produced on a large scale, it would become a vital biomaterial and would be used to create a large variety of medical devices and widely used consumption products. Bacterial cellulose production efficiency should surpass that of timber (forestry) products for the purpose of competing with such sources.

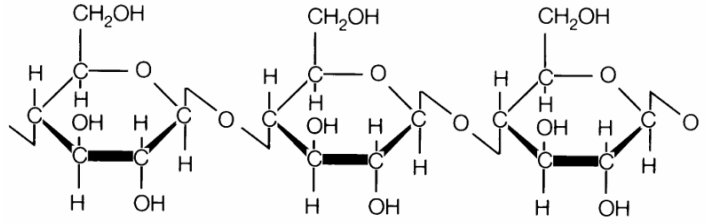

Figure 1: Structural formula of cellulose

Bacterial cellulose (BC) is an exopolysaccharide produced by some bacteria, with unique structural and mechanical properties, presenting high purity when compared to plant cellulose. In the cellulose molecule, the D-glucose rests are joined together by (1,4)β-glycoside bonds. Each monosaccharide residue contains three free hydroxyl groups and therefore, its structure may be displayed as shown Fig. 1.

Cellulose for industrial purposes is usually obtained from plant sources, but cellulose production with bacteria has a great potential. *Acetobacter xylinum*, one of the best bacteria for large scale cellulose production, accepts a wide variety of substrates.

This diversity of utilizable substrates provides considerable flexibility in the location of the manufacturing facility since at least one of these substrates is produced in virtually every region of the world. The availability of inexpensive horticultural products, which have inadequate quality, and of glycerol as a by-product of biodiesel could be a major economic factor in the commercialization of this product. Most importantly, the bacterial cellulose membranes are simple to produce using noncomplex media and protocols.

Romania is an important European producer of apples, having produced 500-550 thousand tons last year. Apples have the third position among the most popular fruit in our country.[16]

This large quantity of apples and glycerol as biodiesel by-product, which are among the most economic carbon sources, could be widely used on a large scale as microbial fermentation substrate for bacterial cellulose production.

The aim of this study is to investigate inexpensive bacterial cellulose production by *A. xylinum* DSMZ-2004 in glass flasks using an extract from poor quality apples and glycerol as a carbon source and optimizing the culture medium by applying the Taguchi method of optimization.

EXPERIMENTAL

Microorganism

Acetobacter xylinum DSMZ-2004 (German Collection of Microorganisms and Cell Cultures), known for its ability to produce cellulose under specific conditions, was used.

Culture maintenance and inoculum

The microorganism was maintained on a Schramm-Hestrin[17] (SH) agar medium composed of (w/v): glucose – 2, peptone – 0.5, citric acid – 0.115, yeast extract – 0.5, Na_2HPO4 – 0.27, storage at +4°C. This culture also constitutes the preinoculum.

Dextrose powder, yeast extract, peptone, anhydrous di-sodium hydrogen phosphate, citric acid monohydrate have been acquired from Scharlau Chemie.

The microorganism presented a compact, white or yellow-white colored aspect. The transfer was performed by washing the surface with sterile distilled water or in nutritional liquid media from which the necessary 1% (v/v) quantity was taken for the next phase. The slants were kept at 5±1 °C until further subcultivation. In the case of cellulose, even from the inoculum phase the formation of a bacterial cellulose pellicle (thin skin) could be observed as the carbon source was exhausted.

The synthesized cellulose pellicle limits the access of the microorganisms to atmospheric oxygen.

The inoculum medium composition was the following (%, w/v): glucose – 1, corn steep liquor – 1, mono potassium phosphate – 0.15, citric acid – 0.1, pH

5. Oxygen supply to the media was achieved by using a working volume of 50 mL/500 mL capacity flask. Inoculum incubation was carried out in a static regime at 30 °C, for 48 hours, when cell concentration reached its maximum.

Culture medium and cultivation conditions

The concentrations of the components were set based on the literature data used for this type of fermentation.[18]

The effect of the factors regarding concentrations of the culture medium components on bacterial cellulose production, using an extract from inadequate quality apples and glycerol, was studied by a Taguchi experimental design. Several media variants have been studied differing in concentration of the mixed carbon source (apple extract – glycerol), nitrogen source (ammonium sulfate) and citric acid.

Volumes of 50 mL culture media prepared in 500 mL Erlenmeyer flasks were inoculated with 5% (v/v) *A. xylinum* DSMZ-2004 inoculum media and were sterilized by autoclavation at 121 ℃, for 15 min. The culture media pH for bacterial cellulose production was between 4.0 and 6.0, cellulose production decreasing at a pH higher than 6.

Incubation was performed under static conditions, at 30 °C, for 14 days.

BC pellicles grown on the liquid surface were collected and washed with water and then immersed in 1 N NaOH for 2 days at 30°C to dissolve the cells included in the pellicle. The pellicles were then immersed in 0.02% NaN_2 solution to reduce microbial contamination and then neutralized with 1% acetic acid and washed with distilled water, successively. They were then dried on a glass plate to determine the weight. The BC production was quantified gravimetrically based on the dry weight of the insoluble BC obtained.

Optimization methodology

The experiments have been designed using an orthogonal matrix L_9 (3^4) with 9 experiments to evaluate the effects of four factors – main carbon source represented by apple extract, secondary carbon source represented by glycerol, nitrogen source represented by ammonium sulfate and citric acid, which showed a significant influence on the bacterial cellulose production, each at three levels (Table 1).

The experiments were carried out according to an experimental design presented in Table 2. Medium composition differed according to the experimental plan in Table 2. Studying the main effects of each factor, the general influence tendencies of these factors on the process can be established. The characteristics can be controlled so that a smaller or greater value in the case of a certain influence factor will lead to the obtainment of the desired result. Therefore, the factor levels that may lead to the best results can be predicted.[19]

Table 1
Control factors and levels for AG (Apples-Glycerol) medium, used in the Taguchi method for optimal bacterial cellulose production by *Acetobacter xyilinum* DSMZ-2004

Factors	Levels		
	1	2	3
A: Glucose (apples), %	2.5	5	7.5
B: Glycerol, %	1	1.5	2
C: $(NH_4)_2SO_4$, %	0.1	0.2	0.3
D: Citric acid, %	0	0.25	0.5

Table 2
L_9 (3^4) Standard orthogonal array

Experiment no.	Factor A	Factor B	Factor C	Factor D
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

Table 3 represents the experimental model design obtained by attributing selected factors and their corresponding levels to the orthogonal matrix L_9 columns. This matrix has 9 lines and 4 columns and each row represents one experimental condition, while each column contains a specific process parameter. More than that, the notation 3^4 implies the fact that more than 4 factors, each at 3 levels (of variation) may be investigated using this orthogonal matrix and their main effects may be estimated considering that all other interactions are negligible, as it is assumed in the present case. The numbers in each column indicate the levels of the specific factors (A, B, C and D). Culture media constituents were the following (g/L): glucose equivalent (apples) – 25, 50, 75; glycerol – 10, 15, 20; ammonium sulfate – 1, 2, 3; citric acid – 0, 2.5, 5.0.

Table 3
Layout of experimental design (3^4)

Experiment no.	A Glucose (apples), %	B Glycerol, %	C $(NH_4)_2SO_4$, %	D Citric acid, %
1	2.5	1	0.1	0
2	2.5	1.5	0.2	0.25
3	2.5	2	0.3	0.5
4	5	1	0.2	0.5
5	5	1.5	0.3	0
6	5	2	0.1	0.25
7	7.5	1	0.3	0.25
8	7.5	1.5	0.1	0.5
9	7.5	2	0.2	0

As glucose source, the inadequate quality apple extract was used. For all of the medium variants, the same magnesium sulfate (0.05%) and yeast extract (0.05%) concentrations were added. The culture media were incubated at 30 °C, for 14 days. The initial medium pH was adjusted to 5.0 with an acetic acid solution and was not controlled later on during the fermentation process.

RESULTS AND DISCUSSION

Three results (corresponding to the three series) for each experimental condition were recorded as presented in Table 4. Conventionally, the data from a designed experiment were used to analyze the average of the objective/response function.

Purified cellulose was obtained, lacking cellular mass and other components from the fermentation broth.

By the Taguchi method, response variation was examined, using an S/N ratio singled out in a corresponding way. In general, S/N represents the ratio between the average value (signal) and the standard deviation (noise). The formula used to calculate the S/N ratio depends on the objective function. Generally, three standard S/N equations are widely used to classify the objective function as: 'larger the better', 'smaller the better', or 'nominal the best'. However, regardless of the type of performance characteristic, a larger S/N ratio is always desirable. In the present study, the bacterial cellulose amount is the 'larger the better' type of quality characteristic, since the goal is to maximize the production. The standard S/N ratio computing formula[20-24] for this type of response is:

$$(S/N)_i = -10\log\left[\frac{1}{n}\sum_{j=1}^{n}\frac{1}{Y_{ij}^2}\right] \quad (1)$$

where i is the number of one trial; Y_{ij} is the measured value of the quality characteristic for the i process and j experiment; n is the number of repetitions for the experimental combination.

The S/N (signal/noise) ratios were computed using equation (1) for each of the nine experimental variants reported in Table 4. After calculating the S/N ratio for each experiment, the S/N average value was calculated for each of the factors and each of the levels. The average value of the S/N ratio for each factor and level was calculated according to equation (2). The average value of the bacterial cellulose concentration for each factor and level was calculated according to equation (3).

$$SN_{A1} = \frac{(S_{N1} + S_{N2} + S_{N3})}{3} \quad (2)$$

$$A_1 = \frac{(A_1 + A_2 + A_3)}{3} \quad (3)$$

Once these S/N ratio values were calculated for each factor and level, they were tabulated as

shown in Table 5, the range R (R = high SN - low SN) of the SN for each parameter was calculated (Table 6). The larger the R value for a parameter, the larger the effect the variable has on the process. This is because the same change in signal causes a larger effect on the variable output being measured.

Since the experimental design is orthogonal, the factor effects can be separated out in terms of S/N ratio and mean response. The average values of the S/N ratios of the four control factors at each of the levels are shown in Figure 2, and the levels corresponding to the highest S/N ratio values were chosen for each parameter representing the optimum condition. Here, the optimum condition corresponds to the maximization of the cellulose production. It is clear from Figure 2 that the optimum levels are as follows (%, w/v): A_3 (apple extract – glucose equivalents: 7.5), B_3 (glycerol: 2), C_2 (ammonium sulfate: 0.2) and D_3 (citric acid: 0.5), respectively.

Table 4
Results for quality characteristics, S/N ratio and bacterial cellulose concentration, g/L

Experiment no.	Series			Average	S/N Ratio
	1	2	3		
1	2.09	2.5	2.3	2.3	7.147
2	6.19	6.3	6.2	6.23	15.895
3	7.67	8.6	7.88	8.05	18.088
4	4.94	5.07	4.98	5.0	13.976
5	2.59	3.61	3.5	3.23	9.897
6	3.45	4.07	3.9	3.8	11.549
7	4.5	5.94	5.2	5.21	14.175
8	6.26	7.5	7.1	6.95	16.771
9	6.86	7.07	6.99	6.97	16.868

Table 5
Average value of S/N ratio and average value response
for each factor and level for AG medium (apple extract–glycerol)

Average value of S/N	Average value response
(S/N) A_1 = 13.71	A_1 = 5.53
(S/N) A_2 = 11.81	A_2 = 4.01
(S/N) A_3 = 15.94	A_3 = 6.38
Optimum level 3 for factor A	
(S/N) B_1 = 11.77	B_1 = 4.17
(S/N) B_2 = 14.19	B_2 = 5.47
(S/N) B_3 = 15.50	B_3 = 6.27
Optimum level 3 for factor B	
(S/N) C_1 = 11.82	C_1 = 4.35
(S/N) C_2 = 15.58	C_2 = 6.07
(S/N) C_3 = 14.05	C_3 = 5.5
Optimum level 2 for factor C	
(S/N) D_1 = 11.30	D_1 = 4.17
(S/N) D_2 = 13.88	D_2 = 5.08
(S/N) D_3 = 16.28	D_3 = 6.67
Optimum level 3 for factor D	

In addition to the S/N analysis, the main effects of the process parameters on the mean response were also analyzed. The mean response refers to the average value of the quality characteristic for each factor at different levels. Thus the average values of the bacterial cellulose production for each factor at the three levels were calculated and plotted in Figure 3.

The mean response analysis (Figure 3) also indicates the same optimum level of the parameters (A_3, B_3, C_2, and D_3), as obtained in the S/N ratio analysis.

The effect of these factors was then calculated by determining the range (Table 6), according to the formula:

Δ = **Max – Min** (4)

It can be noticed that the citric acid had the most significant effect on the process yield, followed by the apple extract, and that all of the other factors, such as ammonium sulfate and glycerol had the lowest effect on the process yield.

Prediction of optimum quality characteristic (QC)

From the analyses of the S/N ratio and the mean response characteristic, the optimum levels of the control factors were determined as: A3, B3, C2, and D3. Hence, the predicted mean of the quality characteristic (cellulose production) was calculated according to equation 5:

$$S_{mp} = Y + (A_3 - Y) + (B_3 - Y) + (C_2 - Y) + (D_3 - Y) \quad (5)$$

where Y is the grand average of the performance/quality/response characteristic (corresponding to all 27 (= 9 x 3) results in Table 4); $\bar{A}_3$, B_3, C_2 and D_3 are the average values of cellulose concentration with the process parameters at their respective optimum levels; S_{mp} indicates the predictable mean of cellulose concentration under optimum conditions.

The values of the averages of different responses were calculated: Y = 5.304 g/L, A3 = 6.38 g/L, B3 = 6.27 g/L, C2 = 6.07 g/L and D3 = 6.67 g/L.

So substituting these in equation (5), the mean optimum value of bacterial cellulose concentration was predicted as: Smp = 9.469 g/L cellulose.

Table 6
Range (R) determination for process factors

Level	S/N A1-A3 (glucose equivalent – apples)	S/N B1-B3 (glycerol)	S/N C1-C3 (ammonium sulfate)	S/N D1-D3 (citric acid)
1	13.71	11.77	11.82	11.304
2	11.81	14.19	15.58	13.88
3	15.94	15.50	14.05	16.28
Δ	4.1	3.7	3.8	4.9
Rank	2	4	3	1

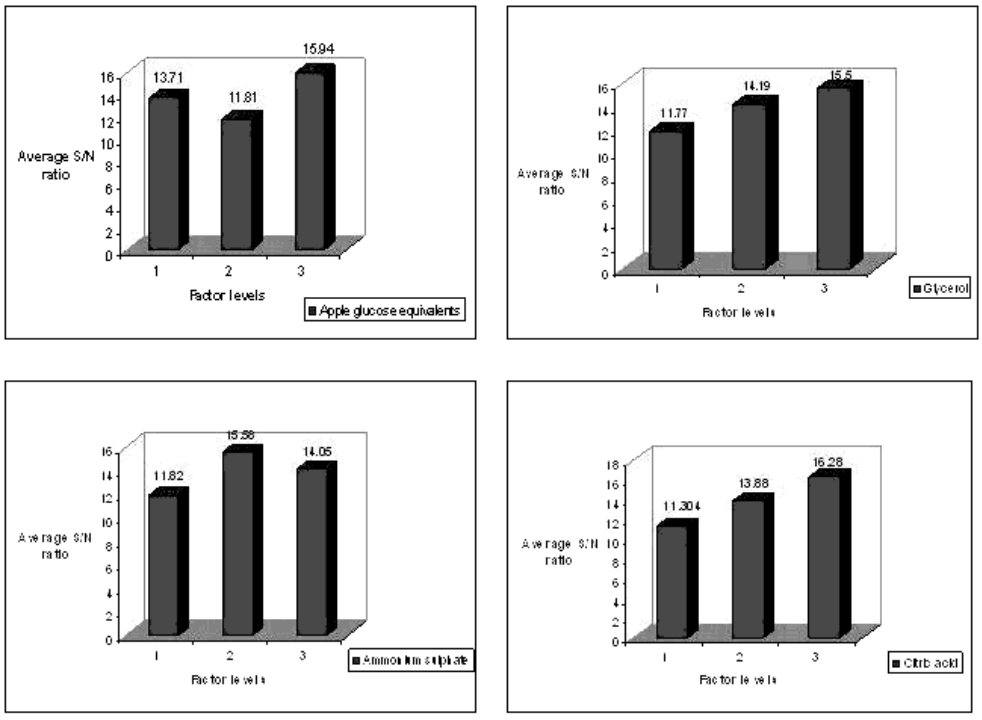

Figure 2: Effects of process parameters on average S/N ratio

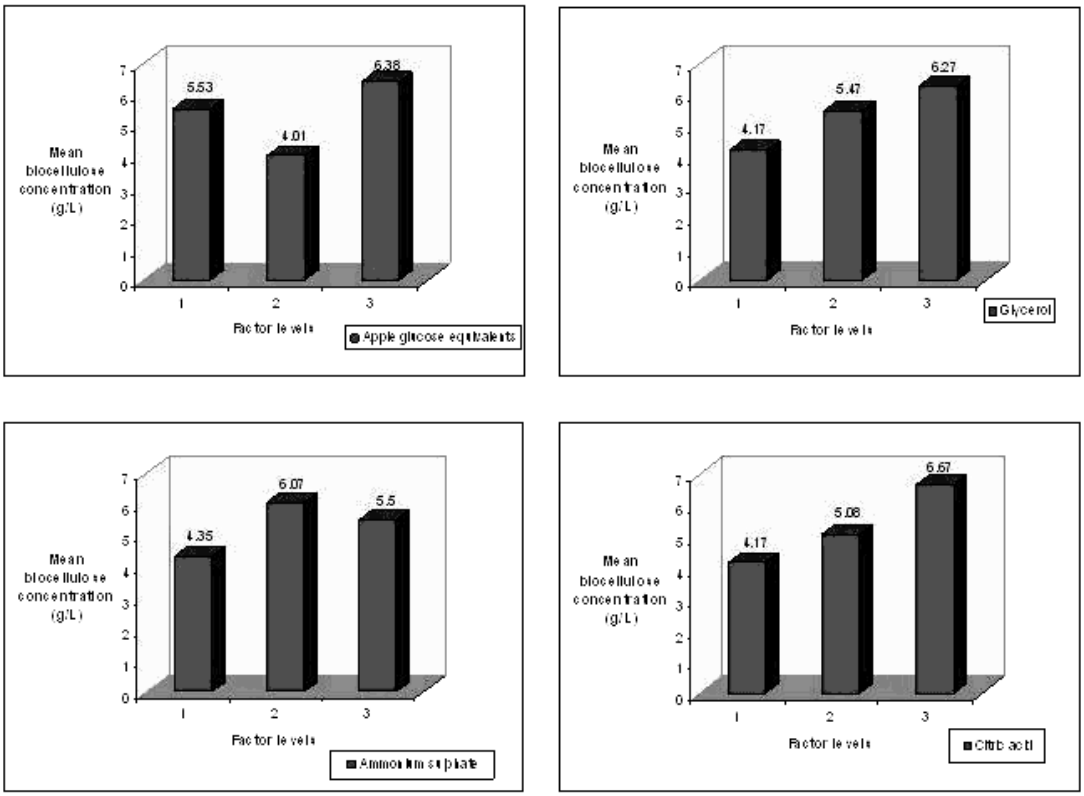

Figure 3: Effects of process parameters on mean response characteristic

CONCLUSION

The optimization of the process parameters for obtaining of bacterial cellulose by enhancing the use value of poor quality horticultural products (apples) as substrate, combined with glycerol, was done using the Taguchi method for parameter design. An orthogonal matrix L_9 format to include 4 control factors each with three levels for the experimental design was used. The process parameters from the biosynthesis medium components selected as factors were glucose equivalent – apples, glycerol, ammonium sulfate and citric acid. Of the 4 process parameters, citric acid and apple glucose had a significant effect on the quality characteristic: bacterial cellulose production. The optimum levels for the process parameters were established as (%, v/v): apple glucose equivalents – 7.5; glycerol – 2.0; ammonium sulfate – 0.2 and citric acid – 0.5.

Under these optimum conditions, the *A. xylinum* DSMZ-2004 cellulose production yield by enhancing the use value of inadequate quality apples in a glycerol combined substrate may be predicted as: Smp = 9.469 g/L. The results of the Taguchi experimental method for cellulose production optimization, by the strain *A. xylinum* DSMZ-2004 showed a cellulose production yield varying from 2.5 to 8.6 g/L, corresponding to the combined effect of the 4 factors at their specific intervals.

The Taguchi experimental model proved to be a good choice for bacterial cellulose biotechnological obtainment procedures by valorization of poor quality horticultural products and glycerol.

REFERENCES

[1] H. Backdahl, G. Helenius, A. Bodin, U. Nannmark, B. R. Johansson *et al*., *Biomaterials*, **27**, 9 (2006).
[2] M. Iguchi, S. Yamanaka and A. Budhiono, *J. Mater. Sci*., **35**, 2 (2000).
[3] D. Klemm, D. Schumann, U. Udhardt and S. Marsch, *Prog. Polym. Sci*., **26**, 9 (2001).
[4] D. Klemm, D. Schumann, F. Kramer, N. Hessler, M. Hornung, H. P. Schmauder and S. Marsch, *Adv. Polym. Sci*., **205**, 49 (2006).
[5] R. P. Chawla, B. Bajaj Ishwar, A. Survase Shrikant and S. Rekha Singhal, *Food Technol. Biotechnol*., **47**, 2 (2009).

[6] N. Hoenich, *BioResources*, **1**, 2 (2006).
[7] W. K. Czaja, D. J. Young, M. Kawecki, R. M. Brown, *Biomacromolecules*, **8**, 1 (2007).
[8] J. D. Fontana, A. M. Desouza, C. K. Fontana, I. L. Torriani, J. C. Moreschi *et al*., *Appl*. *Biochem*. *Biotechnol*., **24**, 25 (1990).
[9] J. D. Fontana, C. G. Joerke, M. Baron, M. Maraschin, A. G. Ferreira *et al*., *Appl*. *Biochem*. *Biotechnol*., **63**, 65 (1997).
[10] A. Okiyama, M. Motoki and S. Yamanaka, *Food Hydrocolloid*., **6**, 6 (1993).
[11] H. Shibazaki, S. Kuga, O. Fumihiko and M. Usuda, *J*. *Appl*. *Polym*. *Sci*., **50**, 965 (1993).
[12] Iuliana Spiridon and V. I. Popa, *Cellulose Chem*. *Technol*., **34**, 275 (2000).
[13] A. Svensson, E. Nicklasson, T. Harrah, B. Panilaitis, D. L. Kaplan *et al*., *Biomaterials*, **26**, 4 (2005).
[14] Y. Z. Wan, L. Hong, S. R. Jia, Y. Huang, Y. Zhu *et al*., *Compos*. *Sci*. *Technol*., **66**, 11-12 (2006).
[15] E. E. Brown, Master of Science Thesis, Washington State University, Department of Chemical Engineering, (2007).
[16] http://faostat.fao.org
[17] M. Schramm, S. Hestrin, *J*. *Gen*. *Microbiol*., **11**, 1 (1954).
[18] K. Watanabe and S. Yamanaka, *Biosci*. *Biotechnol*. *Biochem*., **59**, 1 (1995).
[19] K. Krishna Prasad, S. Venkata Mohan, R. Rao Sreenivas, B. Ranjan Pati, P. N. Sarma, *Biochem*. *Eng*. *J*., **24**, 17 (2005).
[20] A. Ealey Lance, "Quality by Design Taguchi methods™ and US industry", 2nd ed., Sidney, Irwin Professional Publishing, ASI Press, 1994, pp. 189-207.
[21] The Taguchi methodology as a statistical tool for biotechnological applications: A critical appraisal, *Biotechnol*. *J*., **3**, 510 (2008).
[22] D. C. Montgomery, "Design and Analysis of Experiments", 4th ed., New York, John Wiley, 1997.
[23] http://www.slideshare.net/rbalisnomo/Introduction-To-Taguchi-Method-05Sep08
[24]http://controls.engin.umich.edu/wiki/index.php/Design_of_experiments_via_taguchi_methods:orthogonal_arrays