

## Improvement of simple cultivation conditions for polysaccharide synthesis by *Haemophilus influenzae* type b

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*Haemophilus influenzae* type b is a Gram-negative bacterium that causes meningitis infections in infants less than five years old. The capsular polysaccharide of *H. influenzae* type b has been purified and conjugated to a protein to produce a very effective vaccine in children. The price of this vaccine is high because there are many steps of production involved: production and purification of polysaccharide and protein (in general diphtheria or tetanus toxin), chemical conjugation of polysaccharide and protein and finally purification of the conjugated vaccine. Improvement in any of these phases of production will reduce the final price.

Our laboratory studied culture conditions in order to achieve high volumetric production of this polysaccharide allowing, in near future, its introduction in the National Immunization Program with Brazilian technology. We tested three types of batch 1) overlay aeration without pH control; 2) air-sparged with dissolved oxygen tension controlled at 30% without pH control (DOT 30% w/o pH); 3) air-sparged aeration with dissolved oxygen tension controlled at 30% with pH control (DOT 30% +pH).

The biomass and maximum specific growth rate are very similar in the three cases. The cultivations with DOT 30% without pH control showed an average CPS-b production of 574.3 mg/L and the DOT 30% with pH control 943.3 mg/L, accounting for an increase of 36% and 124% over the traditional polysaccharide production with overlay aeration of 420.8 mg/L.

**Keywords:** *Haemophilus influenzae*; overlay aeration; air-sparged aeration; polysaccharide production, pH control.

### 1. Introduction

*H. influenzae* type b is a Gram-negative bacteria with a capsular polysaccharide of repeating units of polyribosylribitol phosphate (CPS-b) and it is the most important factor for its virulence [1, 2]. This bacteria is an important cause of meningitis and others severe infections in children under five years old. The purified CPS-b conjugated to a protein is effective vaccine for children less than five years old [3]. This conjugated vaccine is expensive because there are many steps involved in the production processes. The CPS-b has to be produced and purified; the protein (tetanus toxin or diphtheria toxin) have to be produced purified and inactivated; the CPS-b and the protein is chemically bounded and the new product has to be purified from the reagents. Improvements in any of these steps would contribute to reduce the total cost of production.

The medium culture for *H. influenzae* type b developed by Carty [4] is composed of soy peptone, yeast extract with a culture conditions of 0.25 volume of air per volume of medium (VVM), agitation 200 rpm, without pH control and overlay and sparged aeration. In the american patents added hemin

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and NAD to the medium and 100 rpm agitation without pH control [5, 6]. Recently, Merrit et al. used a medium with casamino acids, yeast extract, NAD and hemin, the pH was controlled at 7.5 and dissolved oxygen tension set at 50% (DOT 50%) with a production of 490 mg/L of polysaccharide.

The purpose of this study is the improvement of the polysaccharide production carried out in three different cultivation conditions and the best process could be used to replace the current one in order to reduce the final cost of vaccine production. This improvement was established with simple methodology like control of dissolved oxygen tension in conjunction with pH control.

## 2. Material and Methods

### 2.1 Bacteria strain

*H. influenzae* type b GB 3291, was obtained from the Centers for Disease Control and Prevention (Atlanta, GA), and the manufacturing master seed was prepared in the Department of Bacteriology at the Instituto Adolph Lutz (São Paulo, SP, Brazil). Preparation of inoculum was done according to M. Takagi *et al.* [7, 8].

### 2.2 Medium Composition and Preparation Medium

The cultivation medium was made according to Takagi et al [8] with modification in the hemin and NAD concentrations: 10.0 g of soy peptone (Difco, Detroit, MI), 5.0 g of dialyzed yeast extract (Merck), 2.5 g of  $K_2HPO_4$  (Merck), 13.1 g of  $Na_2HPO_4$  (Sigma, St. Louis, MO), 3.3 g of  $NaH_2PO_4$  (Sigma), 5.0 g of glucose (Merck), 30 mg of hemin chloride (Sigma), and 15 mg of nicotinamide adenine dinucleotide (NAD) (Sigma) in a final volume of 1 L of distilled water. The pH was adjusted to 7.2 with 5.0 M NaOH. The medium was sterilized by filtration in a Millipore system with a 0.22- $\mu$ m membrane previously autoclaved at 120°C for 15 min and aseptically transferred to the fermentation vessel. UCON LB 652 was used as antifoam. The bioreactor BioFlo 2000 (New Brunswick Scientific Co.) has temperature control (heater/cooler), pH electrode, oxygen probe for measurement of DOT, five peristaltic pumps to add fresh medium, acid or alkali for pH control, anti foam etc,. The bioreactor keeps the temperature, DOT and pH in the elected set values automatically. The DOT probe keeps the desired value by changing the stirring speed, the pH electrode switch-on switch-off the acid/alkali pumps to keep the pH.

### 2.3 Cultivations

The experiments were conducted in a Bioflo 2000 with a 13-L nominal volume, under the following conditions: stirring speed of 100–600 rpm, initial medium volume of 7.4 L, temperature of 37°C and 0.25 VVM of air.

The experiments were conducted as follows: (a) overlay aeration without pH control; (b) air sparged aeration with DOT controlled at 30% of air saturation, without pH control; (c) same as (b) with pH controlled at 7.2. A pulse of glucose to restore the initial concentration of 5g/L was added twice during the batch cultivations when glucose was near depletion.

### 2.4 Biomass concentration

Optical density of the culture was measured at 540 nm using a Ultrospec 100 spectrophotometer (GE Healthcare). Dry cell weight (DCW) was determined in 10-mL samples collected in preweighed tubes. After centrifugation at 3220g and 4°C for 60 min, the pellet was resuspended in 10 mL 0.15 M NaCl and centrifuged again. The centrifuge tube containing the cells was dried at 60°C to achieve constant weight.

### 2.5 Glucose concentration

Glucose concentration was determined in cell-free samples by the glucose oxidase method [9]. The consumed glucose was calculated by the difference between total input and residual glucose present in every sample-collecting time.

### 2.6 CPS-b concentration

Samples of 10mL were withdrawn from bioreactor and centrifuged at  $3220 \times g$ , 4°C for 60min. The supernatant were submitted to dialysis (membrane cut-off 12 000–14 000) against distilled water for 24 h in order to eliminate low molecular weight components from the medium. The polysaccharide concentration was determined using modified Bial method, using ribose (Sigma) as standard [10]. The concentration of CPS-b, polyribosylribitol phosphate, was estimated using a conversion factor in which 1 mg of ribose corresponded to 2.55 mg of polyribosylribitol phosphate. The value for the conversion factor was based on the polyribosylribitol phosphate structural formula reported by Crisel *et al.* [2].

### 2.7 Organic acids concentrations

The metabolic acids produced by *H. influenzae* type b were measured by high-pressure liquid chromatography (HPLC; Shimadzu Co.) with aminex HPX-87H (300mm  $\times$  7.8mm; Bio-Rad) type column, UV detector (210 nm) and an integrator program (class VP, version 6.2; Shimadzu Co.). An aliquot of 20  $\mu$ l of cell-free supernatant, collected during cultivation, was diluted with 0.1 M H<sub>2</sub>SO<sub>4</sub> (1:5), filtered in Millex 0.22  $\mu$ m and injected into the column at 35 °C. A 50 mM H<sub>2</sub>SO<sub>4</sub> solution was used as mobile phase with a flow rate of 0.6 ml/min. Organic acids standard was used to identify and quantified the peak (Bio-Rad Lab. CA, USA No. 125-0586).

### 2.8 Kinetic parameters

The kinetic parameters were estimated based on the results obtained from biomass formation, glucose consumption, cell-free CPS-b and organic acids production in shake flask experiments and batch cultivations. Maximum specific growth rate ( $\mu_{max}$ ), calculated as slope of a plot of time versus Ln of biomass concentration. Yield coefficients  $Y_{P/S}$  and  $Y_{X/S}$ , are the conversion of substrate to products, amount of product (CPS-b) or cells obtained per gram of consumed glucose (S, substrate), calculated as slope of plot of consumed glucose to product or cells. Specific production is amount of product per gram of cells and the productivity amount of product or cells per volume and per time (P/L/h). All parameters were estimated at the end of the growth, when the maximum CPS-b concentration had been reached. The datas collected from the two independent fermentation batches were statistically processed at the 95% confidence interval.

## 3. Results and discussions

The medium of Carty *et al.* [4] showed to be limited for growth of *H. influenzae* type b, Takagi *et al.* [7, 8]. A medium with threefold of NAD (15 mg/L) and hemin (30 mg/L) showed normal exponential growth for *Haemophilus*, (data not show) [7, 8].

The general results of the three experiments are showed in the Table 1. The cell biomass, maximum specific growth rate,  $\mu_{max}$  (1/h) and acetic acid concentration are very similar each other. Metabolites analysis showed that the main product was acetic acid, instead of succinic acids reported by Tuyanu *et al.* [11] and Merrit *et al.* [12].

The Figure 1 illustrates the exponential growth phase lasted 10-12 hours in all experiments. On the other hand, the polysaccharide production showed an improvement with the exponential period for CPS-

b production under DOT 30% without pH control condition was extended about 2 h, consequently, the overall CPS-b production was higher (574mg/L) than in the overlay one (420mg/L). At DOT 30% with pH control, CPS-b production continued throughout the stationary phase, Figure 1, the overall production under pH control within 22 h of cultivation was about 943 mg/L, being significantly higher than the production without pH control. The CPS-b production in the cultures performed without pH control followed cell growth, while the release of polysaccharide to supernatant observed in the experiments with controlled pH took place in both exponential and stationary growth phases.

Table 1. Kinetics parameters

	Overlay	DOT 30% air saturation without pH control	DOT 30% air saturation with pH control
Maximum biomass concentration, (g DCW/L)	4.3	3.3	3.1
Maximum CPS-b concentration (mg/L)	420.8	574.3	943.3
Maximum acetic acid concentration (g/L)	7.2	7.7	9.3
Polysaccharide yield coefficient ( $Y_{P/S}$ )	48.8	55.1	68.3
Cell yield coefficient ( $Y_{X/S}$ )	0.39	0.29	0.22
Specific production ( $SP_{P/X}$ )	126.0	193.7	312.3
Overall CPS-b productivity (mg CPS-b/L/h)	28.0	29.3	41.0
Maximum specific growth rate, $\mu_{max}$ (1/h)	0.38	0.35	0.34

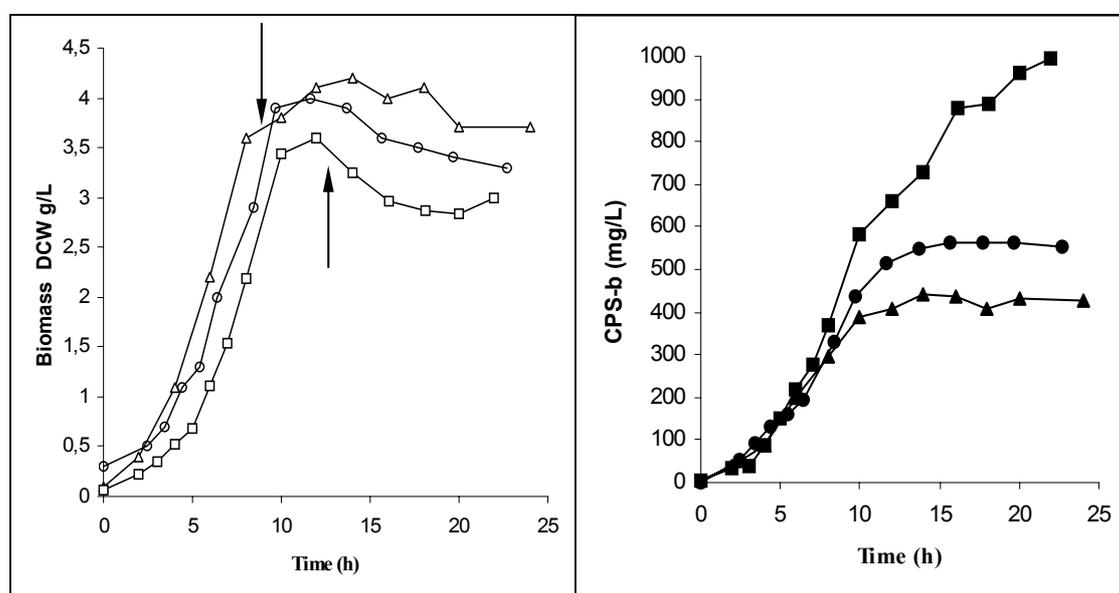
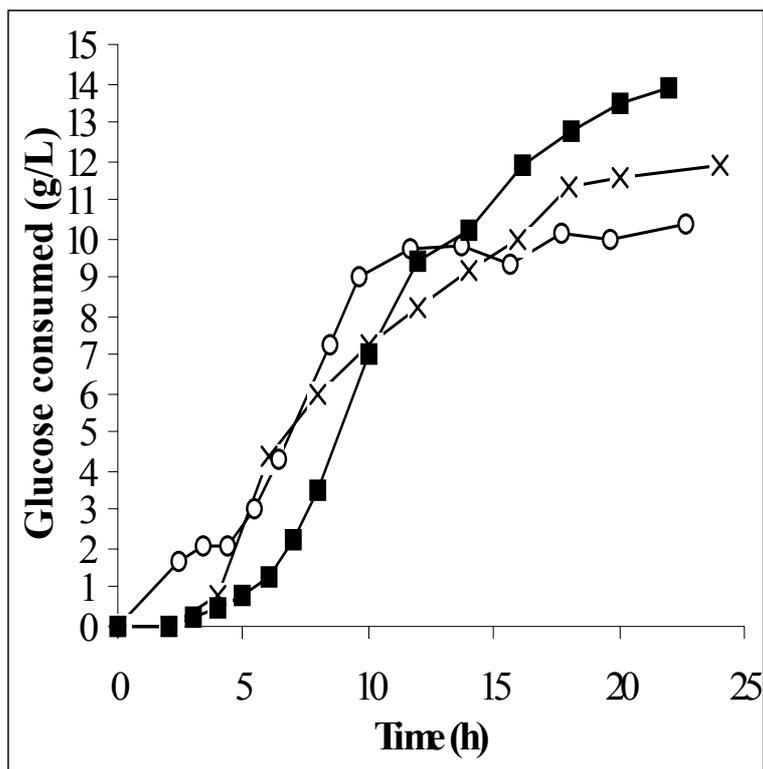


Figure 1. A) time course of growth of *H. influenzae* type b:  $\Delta$  overlay aeration without pH control;  $\circ$  DOT 30 % air saturation without pH control;  $\square$  DOT 30 % air saturation with pH control. B) time course of CPS-b production:  $\blacktriangle$  overlay aeration without pH control;  $\bullet$  DOT 30 % air saturation without pH control;  $\blacksquare$  DOT 30 % air saturation with pH control. Arrows indicate glucose addition time.

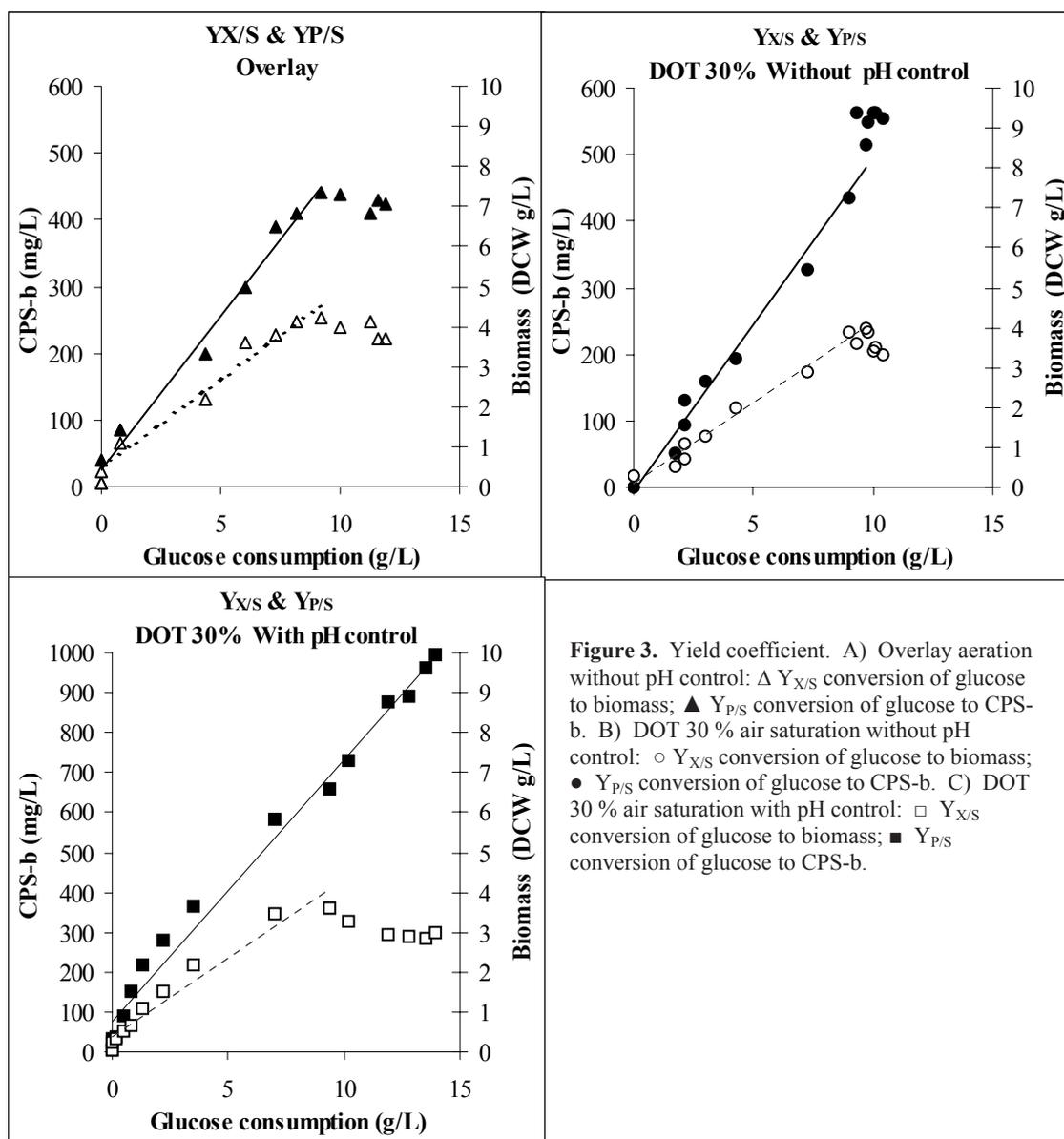
The time course of glucose consumption followed the pattern showed in the Figure 2. The cultivation with overlay consumed a total of 11.9 g/L (79%) and the DOT 30% without pH control had a total of 10.4 g/L (69%). The consumption of glucose with pH control showed two phases one up to 10 h with fast consumption of glucose concomitant with exponential cell growth and CPS-b synthesis and second with slow rate of consumption with stationary phase of cell growth but with a vigorous synthesis of polysaccharide, the total consumed glucose was 13.9 g/L (93%). The total glucose added for the three conditions was 15 g/L.



**Figure 2.** Time course of glucose consumption. X overlay aeration; O DOT 30% without pH control; ■ DOT 30% with pH control.

Yield coefficients  $Y_{X/S}$  and  $Y_{P/S}$ , g cell or mg of CPS-b produced per g of substrate consumed (glucose) showed some differences. In overlay experiments showed that conversion of glucose to cells and CPS-b stopped around 9.2 g/L of consumed glucose, but the glucose was consumed up to 11.9 g/L without conversion, Figure 3A. The culture with DOT 30% and without pH control stopped the conversion of substrate to cells after 9.7 g/L but the synthesis of polysaccharide went for a little more time, Figure 3B.

In the other hand the cultivation with DOT 30% and pH control showed an interesting pattern. The cells consumed 13.9 g from a total of 15g but in two different ways. There was a good conversion of glucose to polysaccharide in Figure 3C can be observed a straight line of conversion of glucose to polysaccharide. The conversion of substrate to cells stopped at 9.4 g of consumed glucose but the consumption of glucose went off but directed to the synthesis of polysaccharide, as illustrated in Figure 3C.



**Figure 3.** Yield coefficient. A) Overlay aeration without pH control:  $\Delta$   $Y_{X/S}$  conversion of glucose to biomass;  $\blacktriangle$   $Y_{P/S}$  conversion of glucose to CPS-b. B) DOT 30 % air saturation without pH control:  $\circ$   $Y_{X/S}$  conversion of glucose to biomass;  $\bullet$   $Y_{P/S}$  conversion of glucose to CPS-b. C) DOT 30 % air saturation with pH control:  $\square$   $Y_{X/S}$  conversion of glucose to biomass;  $\blacksquare$   $Y_{P/S}$  conversion of glucose to CPS-b.

Improvements of the CPS-b production, polyribitol phosphate, was carried out with three important modifications in the method of Carty *et al* [4]: medium composition, sparged aeration and pH control.

A medium with threefold of growth factors (NAD, hemin) showed a normal exponential growth for *H. influenzae*, (data not show) [7, 8].

Sparged aeration without pH control improved the CPS-b production by 36% with respect to the overlay by a extension of two hours the time course for synthesis. The polysaccharide yield coefficient  $Y_{P/S}$  and specific production  $SP_{P/X}$  increased 13% and 53.7% respectively with sparged aeration without pH control and the CPS-b production followed cell growth [7].

In the experiments with pH control, the maximum CPS-b concentration and  $SP_{P/X}$  were more than double in comparison with overlay conditions, the  $Y_{P/S}$  and overall productivity increased around 40%. Another interesting observation was that with pH control CPS-b production continued through the

stationary phase, the release of polysaccharide took place in both exponential and stationary growth phases, a change from cell growth associated to a partially cell growth non-associated [7, 13].

The influences of pH on polysaccharide and metabolites production can result in changes in polysaccharide composition such as described in different bacteria: *Propionibacterium microaerophilum* [14], *Azospilillum brasiliense* [15] or *Lactobacillus helveticus* [16]. Furthermore, production of CPS from *L. helveticus* was increased in acidic environment [16].

However, several genes have been shown to be involved in the regulation and synthesis of bacteria polysaccharides [17, 18, 19]. It is therefore possible that the pH and the level of oxygen could affect gene expression and/or regulation of enzymes involved in synthesis of the polysaccharide.

In conclusion, by change in concentration of growth factors, control of the pH and dissolved oxygen tension, the production of PRP was significantly enhanced. These modifications do not require additional reagents or equipment, the present data would provide an important contribution for large scale processes without elevating the costs.

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## References

- [1] C.W. Norden in Bacterial infections of humans, ed by A.S. Evans and H.A. Feldman. Plenum, New York, (1982), pp 259–273.
- [2] R.M. Crisel, R.S. Baker and D.E. Dorman. Journal of Biological Chemistry **250**, 4926, (1975).
- [3] J.D. Wenger. Pediatric Infection Diseases Journal **17**, S132, (1998).
- [4] C.E. Carty, R. Mancinelli, A. Hagopian, F.X. Kovach, E. Rodriguez, P. Burke, N.R. Dunn, W.L. McAleer, R.Z. Maigetter and P. Kniskern. Developments in Industrial Microbiology **26**, 763, (1985).
- [5] M.R. Hilleman, J.Y. Tai and R.L. Tolman. US Patent 4 459 286 (1984).
- [6] S. Marburg, P.J. Kniskern and R.L. Tolman. US Patent 4 882 317 (1989).
- [7] M. Takagi, J. Cabrera-Crespo, J. Baruque-Ramos, I. Raw, T.C. Zangirolami and M.M. Tanizaki. Applied Biochemistry and Biotechnology **110**, 91, (2003).
- [8] M. Takagi, J. Cabrera-Crespo, T.C. Zangirolami, I. Raw and M.M. Tanizaki. Journal of Chemical Technology and Biotechnology **81**, 182, (2006)
- [9] A. Dahlqvist. Biochemical Journal **80**, 547, (1961).
- [10] G. Ashwell in Methods of Enzymology, Vol. III, edited by S.P. Colwick and N.O. Kaplan. Academic Press, New York, USA, p 87–90, (1957).
- [11] J.E. Tuyau, W. Sims and R.A.D. Williams. Journal of General Microbiology **130** (1), 787, (1984).
- [12] J. Merrit, G. Allard, L. O'Toole, R. Swartz and P. Licari. Journal of Biotechnology **81**, 189, (2000).
- [13] P. Anderson. Infection and Immunity **13**, 581, (1976).
- [14] M. Koussémon, Y. Combet-Blanc, B. Ollivier. Current Microbiology **46**, 141, (2003).
- [15] S.A. Konnova, Y.P. Fedonenko, O.E. Makarov, V.V. Ignatov. Biology Bulletin **30**, 430, (2003).
- [16] M.I. Torino, M.P. Taranto, F. Sesma, G. Font de Valdez. Journal of Applied Microbiology **91**, 846, (2001).
- [17] S.A. Morse, B.H. Hebel. Infection and Immunity **21**, 87, (1978).
- [18] J.P. Dillard, M.W. Vandersea, J. Yother. Journal of Experimental Medicine **181**, 973, (1995).
- [19] J.R. Gilsdorf. Infection and Immunity **66**, 5053, (1998).