J Ind Microbiol Biotechnol (2012) 39:897–908
DOI 10.1007/s10295-012-1099-8

Optimization of medium formulation and seed conditions for expression of mature PsaA (pneumococcal surface adhesin A) in *Escherichia coli* using a sequential experimental design strategy and response surface methodology

Ariane Leites Larentis · Júlia Fabiana Monteiro Quintal Nicolau · Ana Paula Corrêa Argondizzo · Ricardo Galler · Maria Isabel Rodrigues · Marco Alberto Medeiros

Received: 15 September 2011 / Accepted: 28 January 2012 / Published online: 25 February 2012
© Society for Industrial Microbiology and Biotechnology 2012

Abstract PsaA, a candidate antigen for a vaccine against pneumonia, is well-conserved in all *Streptococcus pneumoniae* serotypes. A sequence of two-level experimental designs was used to evaluate medium composition and seed conditions to optimize the expression of soluble mature PsaA in *E. coli*. A face-centered central composite design was first used to evaluate the effects of yeast extract (5 and 23.6 g/L), tryptone (0 and 10 g/L), and glucose (1 and 10 g/L), with replicate experiments at the central point (14.3 g/L yeast extract, 5 g/L tryptone, 5.5 g/L glucose). Next, a central composite design was used to analyze the influence of NaCl concentration (0, 5, and 10 g/L) compared with potassium salts (9.4 g/L K_2HPO_4/2.2 g/L KH_2PO_4), and seed growth (7 and 16 h). Tryptone had no significant effect and was removed from the medium. Yeast extract and glucose were optimized at their intermediate concentrations, resulting in an animal-derived material-free culture medium containing 15 g/L yeast extract, 8 g/L glucose, 50 μg/mL kanamycin, and 0.4% glycerol, yielding 1 g/L rPsaA after 16 h induction at 25°C in shake flasks at 200 rpm. All the seed age and salt conditions produced similar yields, indicating that no variation had a statistically significant effect on expression. Instead of growing the seed culture for 16 h (until saturation), the process can be conducted with 7 h seed growth until the exponential phase. These results enhanced the process productivity and reduced costs, with 5 g/L NaCl being used rather than potassium salts.

Keywords Design of experiments (DoE) · Modeling · Optimization · Response surface methodology (RSM) · Statistical experimental design

A. L. Larentis (✉) · J. F. M. Q. Nicolau · A. P. C. Argondizzo · R. Galler · M. A. Medeiros
Laboratório de Tecnologia Recombinante (LATER), VDTEC (Vice-Diretoria de Desenvolvimento Tecnológico), Bio-Manguinhos/Fundação Oswaldo Cruz (Fiocruz), Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ 21040-360, Brazil
e-mail: ariane@bio.fiocruz.br; ariane@peq.coppe.ufrj.br

M. I. Rodrigues
Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas (UNICAMP), Cidade Universitária Zeferino Vaz, Barão Geraldo, Campinas, SP 13083-970, Brazil

M. I. Rodrigues
PROTIMIZA Consulting and Training in Experimental Design and Process Optimization, Campinas, SP, Brazil
URL: http://www.protimiza.com.br

Introduction

Pneumococcal surface adhesin A (PsaA) is a 37-kDa cell membrane-associated, surface-exposed lipoprotein, which is hydrophobic, immunogenic, genetically conserved, and detected on all different known *Streptococcus pneumoniae* serotypes (>90 isolates). PsaA is essential for the virulence of this species [7, 24]. The immunogenic lipoprotein PsaA is a potential candidate antigen for the development of a protein vaccine capable of inducing serotype-independent protection against *S. pneumoniae* [1, 2, 8, 24], owing to its potential to reduce nasopharyngeal colonization, its high degree of conservation, and its widespread occurrence among the different pneumococcal serotypes. PsaA is also an important pneumococcal vaccine target because to induce herd immunity against *S. pneumoniae*, it is crucial to elicit protection against carriage, as pneumococcal infection is usually acquired from carriers rather than from infected individuals [2, 3, 7, 10]. Several studies employing different vaccine strategies have indicated that PsaA is

effective in preventing colonization and is protective against carriage; studies of the immunization of mice with PsaA and other antigens have presented promising protective results [3, 7, 10, 22, 26, 28]. These proteins might serve as a vaccine by themselves, but it is more likely that they would be more effective in conjunction with polysaccharide–protein conjugates or as carriers for polysaccharides [28] or other proteins or adjuvants, stimulating immune responses to non-immunogenic antigens [8]. The expensive commercially available vaccines are based on polysaccharides and the protection is limited in young children and elderly, the group most susceptible to pneumococcal diseases; conjugated vaccines induce immunity against limited specific serotypes [1, 2].

PsaA has a hydrophobic signal peptide and a transmembrane region at the N-terminal sequence. The cleavage of this signal sequence results in a mature protein with 32.5 kDa. Our previous results indicated that expression in *E. coli* of the PsaA with the hydrophobic leader sequence was lower than the expression of mature PsaA [14]. On the basis of the results indicating PsaA as a potential vaccine candidate, the gene corresponding to mature PsaA from *S. pneumoniae* serotype 14, the most prevalent in Brazil [15], was previously cloned by our research group in a kanamycin-resistant plasmid, without purification tags [14]. The optimization of the induction conditions (isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration, temperature, and time), with a view to enhancing mature PsaA (rPsaA) yields and reducing process costs, was performed using experimental design techniques and resulted in the expression of the recombinant protein at 25°C for 16 h, with 0.1 mM IPTG in Terrific Broth (TB) medium supplemented with 1% glucose, 0.4% glycerol, and 50 µg/mL kanamycin. Under these conditions, around 0.8 g mature rPsaA/L per culture medium was expressed in *E. coli* BL21 Star™ (DE3) by pET28a induction with IPTG, corresponding to around 30–35% of the total protein [14].

To develop a process for the purposes of mature rPsaA production in *E. coli*, the next objective was to study a cheaper process by optimizing the medium composition to substitute TB medium. In particular, the removal of tryptone from the culture medium formulation was evaluated for the purposes of compliance with health agency regulations, because tryptone is obtained from the breakdown of bovine casein by trypsin, which is an animal source. Seed conditions were also evaluated. Considering these aspects, in this work, the medium formulation (yeast extract, tryptone, glucose, and salt concentrations) and seed conditions were optimized using experimental design to reduce the cost of the production of mature rPsaA in *E. coli* for use as a potential candidate in a vaccine against pneumonia.

Materials and methods

Plasmid and expression system

The mature PsaA gene (870 bp) from *S. pneumoniae* serotype 14 was cloned into the pET28a expression vector (Novagen, EMD Chemicals, Inc., Gibbstown, NJ, USA) with IPTG induction. *E. coli* BL21 Star™ (DE3) (Invitrogen, Carlsbad, CA, USA) was used as the expression host. Cloning techniques and chemicals were reported in Larentis et al. [14].

Cell viability determination

The viability of cells from stocks of recombinant *E. coli* BL21 Star™ (DE3)/pET28a/*psaA* in LB (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl, pH 7 adjusted with NaOH) with 25% glycerol stored at −70°C was evaluated by counting colony forming units (CFU). Serial dilutions were performed in PBS pH 7.4 (8 g/L [137 mM] NaCl, 0.2 g/L [2.7 mM] KCl, 1.44 g/L [10 mM] Na_2HPO_4, 0.24 g/L [2 mM] KH_2PO_4). A 10-µL aliquot of the sample diluted from 10^6- to 10^{10}-fold was plated in LB agar (LB broth with 1.5% (w/v) agar) with 50 µg/mL kanamycin, after which the plates were incubated for around 18 h at 37°C to obtain isolated colonies. From the glycerol stock containing 2.2×10^{11} CFU/mL (with around 15% error in counting analysis), 10 µL was inoculated.

Expression

Recombinant *E. coli* BL21 Star™ (DE3)/pET28a/*psaA* was cultivated in 10 mL seed culture with inoculation of 10 µL of cells from the glycerol stock in TB medium (23.6 g/L yeast extract, 11.8 g/L tryptone, 9.4 g/L potassium phosphate dibasic [K_2HPO_4], 2.2 g/L potassium phosphate monobasic [KH_2PO_4], pH 7.2) supplemented with 1% glucose, 0.4% glycerol, and 50 µg/mL kanamycin at 37°C and 200 rpm in 50-mL Erlenmeyer flasks. The seed culture was grown for 16 h until the stationary phase (absorbance at 600 nm near 5).

The seed culture was stored at 4°C for 4–5 h to allow for expression to be performed overnight, using the following procedure: 2% of this saturated seed culture was diluted in 100 mL media of different compositions in 500-mL Erlenmeyer flasks, resulting in an initial absorbance at 600 nm of around 0.1. Cells were grown at 37°C and 200 rpm until the exponential phase was reached (Abs_{ind} 0.8–0.9), at which point 0.1 mM IPTG was added. After IPTG induction, mature rPsaA was expressed at 25°C for 16 h. Each medium had its yeast extract, tryptone, and glucose content varied according to the experimental design and all the media were supplemented with

0.4% glycerol and 50 μg/mL kanamycin. The expression level after 16 h induction was compared with the total protein extract obtained from the cells before IPTG induction (uninduced samples). *E. coli* BL21 Star™ (DE3)/pET28a (plasmid without any gene inserted in the cell) was used as a negative control. The culture medium obtained from the supernatant after 16 h induction was used for the determination of pH and glucose concentration, as described below.

Cell growth, glucose concentration, and pH measurements

Cell growth was measured by absorbance at 600 nm using a Thermo Genesys spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The glucose concentration was determined by an enzymatic colorimetric method using glucose oxidase (LABORLAB, Guarulhos, SP, Brazil) and analyzed by absorbance at 500 nm. Samples were diluted tenfold, when necessary, in order to ensure reaction linearity (until 450 mg/dL, according to the manufacturer). The pH after 16 h induction was determined using standard solutions at pH 4.0 and 7.0. The initial pH for all the culture media tested was 7.0.

Quantification of rPsaA expression by densitometry analysis

The pellets from 1-mL expression samples (uninduced and after 16 h induction) harvested by centrifugation were resuspended in 20 mM Tris and 1 mM EDTA (pH 8) buffer according to the ratio of 250 μL buffer to each $Abs_{600nm} = 1$. Cell disruption for preparation of the total protein extract and soluble fraction was performed according to that previously reported by Larentis et al. [14]. The mature rPsaA expression was evaluated using total protein extract samples by densitometry analysis of the band areas from the SDS-PAGE corresponding to 32.5 kDa and calculated by comparison with a molecular weight standard, which yielded bands with similar intensity to rPsaA. Different kinds of proteins were used in the standard marker to minimize errors associated with different Coomassie Blue binding characteristics. Also, a standard curve was run from 1 to 15 μg BSA in SDS-PAGE (using 10-μL samples) to confirm the intensity of the bands and evaluate the errors associated with this technique. The errors calculated from the comparison of the nominal values with the calculated values were greater than 20% for samples of over 6 μg and 30% for samples of over 8 μg. For this reason, the bands relating to 16 h induction were considered until 5 μg for the calculations in the densitometry analysis, whose absorbance measurement could be used in the Beer–Lambert law to determine protein concentration.

Experimental design for evaluation of medium composition and statistical analysis

The analysis of the effects of three independent variables in the medium composition (yeast extract, tryptone, and glucose concentrations) on the expression of soluble mature rPsaA in *E. coli* BL21 Star™ (DE3)/pET28a/*psaA* was performed using face-centered central composite experimental design. The statistical evaluation of the effects of the composition medium variables on the expression of rPsaA, the response surface methodology (RSM), and the analysis of variance (ANOVA) were performed with the help of the STATISTICA 9.1 software (StatSoft, Inc., Tulsa, OK, USA) using the coded variable at levels −1 (lowest value of the experimental conditions used), +1 (highest value of the experimental conditions), and 0 (central point condition, defined as the intermediate value in the range of each variable), which are described in Table 1 (first design).

By normalizing each variable under analysis, their effects can be compared without the influence of the magnitude of the interval employed for each one. The experiments at the central point condition (0) of all the variables were performed to analyze experimental error and to check the curvature of the responses. The replicates at the central point were used to calculate the average and standard deviation for each response (cell growth, protein expression, glucose concentration, and pH). The significance of each linear and quadratic coefficient and interaction was determined using Student's t test at a 0.05 probability level (95% confidence level). The effects were statistically significant when the p value was less than 0.05. For the statistical evaluation of rPsaA expression, the variables with p values no greater than 0.15 (85% confidence level) were considered statistically significant because of the variability associated with the recombinant expression process and analytical methods involved. Every time a different set of runs of the experimental design was carried out, one or two assays at the central point conditions were added, so that the statistical analysis could give a more realistic assessment of the experimental error inherent to the process.

A second-order polynomial model including all the linear, quadratic, and linear interaction coefficients was used to calculate the predicted response, as indicated in the equation below, where x_1, x_2, and x_3 are the coded values of independent variables, b_0 represents the medium/intersection, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, b_{ij} the linear interaction in the model, and Y is the dependent variable of interest designed to optimize the medium formulation with the best levels of mg rPsaA/L:

$$Y = b_0 + b_1x_1 + b_{11}x_1^2 + b_2x_2 + b_{22}x_2^2 + b_3x_3 + b_{33}x_3^2 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

Table 1 Values and corresponding coded levels (−1, 0, and +1) of independent variables for medium composition used in the face-centered central composite experimental design for three variables (first design) and for two variables in a central composite experimental design (second design)

Variable	Code	−1	0	+1
First design				
Yeast extract	x_1	5 g/L	14.3 g/L	23.6 g/L
Tryptone	x_2	0 g/L	5 g/L	10 g/L
Glucose	x_3	1 g/L	5.5 g/L	10 g/L
Second design				
NaCl	–	0 g/L	5 g/L	10 g/L
Seed growth[a]	–	7 h (exponential phase)	7 h 16 h	16 h (stationary phase)

[a] The central level (0) corresponds to an intermediate value between the −1 and +1 interval of each variable, except for the seed growth age, as explained in "Materials and methods"

Validation of experimental design results for medium optimization

The experimental design results were validated in triplicate at the highest rPsaA expression levels using a less expensive culture medium whose composition was reached by model analysis (15 g/L yeast extract, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 8 g/L [0.8%] glucose, 50 μg/mL kanamycin, and 0.4% glycerol) at 25°C, 200 rpm, 16 h induction with 0.1 mM IPTG. Tryptone was not included in the medium.

Evaluation of the effects of salt and seed conditions

The culture medium defined by model analysis (15 g/L yeast extract, 8 g/L [0.8%] glucose, 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, initial pH 7) was compared with different salt conditions (10 g/L NaCL, 5 g/L NaCl, and no salt) rather than potassium salts, and with the same yeast extract and glucose concentrations. The culture medium was supplemented with 50 μg/mL kanamycin and 0.4% glycerol. In order to reduce the process time, the seed culture grown to saturation (16 h) was compared with seed growth for 7 h until it reached the exponential phase (absorbance at 600 nm in the range of 2.5–3), when the cells were inoculated for the expression assays. Expression was performed as described in the "Expression" section.

The effect of NaCl concentration and seed growth on the expression of soluble mature rPsaA was analyzed using a central composite design, containing all the combinations of the two variables and the central conditions in duplicate, as described for the second design in Table 1. In this experimental design, the time used for the second variable was related to the age of the seed culture: condition −1 corresponded to growth until the exponential phase, whereas +1 related to the stationary growth phase (Table 1). In this way, samples were not taken during seed growth, as warned against by Rodrigues and Iemma [25] for batch systems, thus avoiding having time as an independent variable. This is the reason why the central condition was performed in two different runs: 5 g/L NaCl and 7 h (growth until exponential phase) and 5 g/L NaCl and 16 h (seed growth until stationary phase).

The statistical analysis was performed in the same way as it was for the medium variables, with the effects being considered statistically significant when the p value was less than 0.05. In this experimental design, only linear effects and linear interactions could be evaluated.

Confirmation of optimized conditions comparing 100- and 400-mL shake flasks

The optimized culture medium and seed conditions were tested for the same volume at which the experimental design was carried out (100-mL culture volume in 500-mL Erlenmeyer flasks) and also at a four times greater volume (400-mL culture volume in 2-L Erlenmeyers).

Results and discussion

An efficacious and cheaper vaccine against pneumococcal diseases is one of the priorities for the public health system in Brazil [14]. In this work, a sequence of experimental designs was used to optimize the process conditions for the expression of recombinant mature PsaA from *S. pneumoniae* in *E. coli*. The analysis of the conditions for the expression of rPsaA was performed to attain higher protein yields, reduce production costs, and fulfill regulatory requirements for the large-scale production of this potential vaccine candidate at Bio-Manguinhos/Fiocruz. The strategy employed in this work was to evaluate these conditions in shake flasks, and to use experimental design and

statistical tools to evaluate the conditions for scaling up the optimized process in a bioreactor in future studies.

Preliminary results in Luria–Bertani (LB) medium indicated that both the expression of mature rPsaA per cell and cell growth were lower in the LB medium than they were in the TB medium, i.e., that the yield of soluble recombinant protein was higher in the richer medium (TB). However, higher process variability was verified in TB when different yeast extract and tryptone batches were compared, because of the high concentration of complex components in this medium and the batch-to-batch variations inherently associated with these ill-defined components [9]. In addition, TB is more expensive than LB. Also, similar expression levels of mature rPsaA were achieved using 10 g/L NaCl rather than potassium salts with the same yeast extract and tryptone concentrations in the TB culture medium. Similarly, when the LB medium was buffered with potassium salts, expression was found to be in the same range as the expression obtained in LB with NaCl. On the basis of these preliminary data, experimental design tools were used to optimize the medium formulation in the production of mature rPsaA in *E. coli* to obtain similar expression levels using a poorer culture medium as had been obtained in the soluble fraction using TB in a bid to evaluate:

1. The influence of the yeast extract concentration comparing 5 g/L used in the LB medium (−1) with 23.6 g/L used in the TB medium (+1), and 14.3 g/L at the central point (0)
2. The removal of tryptone (−1) from the culture medium, in comparison with using 10 g/L tryptone (+1) (a similar concentration to that used in the LB and TB culture media), and 5 g/L at the central point (0)
3. The influence of glucose concentration in the medium: 1 g/L or 0.1% (−1), 10 g/L or 1% (+1), and 5.5 g/L or 0.55% at the central point (0)
4. NaCl, the salt present in LB medium, in different concentrations: 0 g/L (−1), 5 g/L (central point), and 10 g/L (+1), and compare with the potassium salts (9.4 g/L K_2HPO_4 and 2.2 g/L KH_2PO_4) present in TB
5. Seed conditions for the inoculation of *E. coli* BL21 Star^{TM} (DE3)/pET28a/*psaA* in the shake flask experiments in order to reduce the process time, comparing seed growth for 16 h until the stationary phase (+1) with 7 h growth until the exponential phase (−1)

In order to evaluate the effects of yeast extract, tryptone, and glucose on mature rPsaA expression, a face-centered central composite design was employed. Once it had been selected what yeast extract, tryptone, and glucose concentrations would be used in the culture medium formulation, the influence of NaCl instead of potassium salts was tested, as was the inoculation of the *E. coli* culture at the exponential phase rather than a saturated culture (seed growth conditions), in order to optimize mature rPsaA production.

As discussed by Lee et al. [16], much effort has been expended to improve recombinant expression systems by manipulating the genetic factors; however, studies into the expression of recombinant proteins usually fail to consider the effects of the composition of the medium in which cell growth and protein synthesis occur, despite research on microbial culture in the biotechnology industry for several decades having made it clear that there is no universal medium because cells generated by cloning have phenotypes that are physically and physiologically different. The productivity of a protein is greatly affected by the nutrient composition of the medium [6]. Also, it is well known that the production of secondary metabolites in bacterial strains depends on the composition of the medium and that a high concentration of a certain component may inhibit the synthesis of a specific metabolic formulation required for protein production [16]. Because of the large number of process variables and the metabolic complexity of microorganisms, the evaluation of the medium composition is often overlooked. When it is studied, the most common strategy is to evaluate the influence of the different variables by changing them one at a time, failing to take experimental error into account or the interactions between the individual medium components and the microbial metabolism, possibly leading to misinterpretation of the data. This is also time-consuming, especially when there are many variables involved [6, 11, 13, 16, 23]. As no single set of culture conditions can be applied to different kinds of cells, a screening and development approach is necessary for optimal performance and for reducing time and costs. Statistical optimization and experimental design have proved to be very effective when several variables have to be considered and the relative importance of each one is not yet known [16, 30]. Experimental design can be used to evaluate which variables and which variable interactions influence recombinant protein expression, information that cannot be obtained from methods that vary one variable at a time. Recently, researchers have given more attention to the effect of culture medium on protein expression, and more articles have been published that make use of experimental design tools. Experimental design has been used by other research groups to optimize the culture medium composition for recombinant protein expression in *E. coli* [4, 6, 9, 11, 16, 19, 20, 23, 29, 30] or seed conditions and inoculum level/density [11, 21, 27].

Statistical analysis of the effects of yeast extract, tryptone, and glucose

Under all the expression conditions, including the different yeast extract, tryptone, and glucose concentrations, the

Table 2 Results of face-centered central composite experimental design for evaluation of the effects of yeast extract, tryptone, and glucose concentrations on media formulation with 9.4 g/L K_2HPO_4, 2.2 g/L KH_2PO_4, 50 μg/mL kanamycin, and 0.4% glycerol. Comparison between experimental and predicted rPsaA expression data (mg rPsaA/L)

Run[a]	x_1 (yeast extract)	x_2 (tryptone)	x_3 (glucose)	Cell growth (Abs_{600nm})	Residual glucose (g/L)	Final pH	rPsaA (mg/L)	Predicted (model)[b]	Relative error (%)[c]
1	−1	−1	−1	4.57	0.0	6.75	505	435	13.8
2	1	−1	−1	4.89	0.0	6.86	715	732	−2.4
3	−1	1	−1	5.22	0.0	6.92	561	604	−7.6
4	1	1	−1	5.70	0.0	6.90	807	715	11.4
5	−1	−1	1	5.41	3.8	6.32	595	639	−7.3
6	1	−1	1	7.58	2.3	6.01	1,179	1,088	7.7
7	−1	1	1	6.42	4.0	6.41	721	656	9.1
8	1	1	1	6.51	3.2	6.12	898	919	−2.4
9	0	0	0	7.33	0.2	6.20	876	842	3.9
10	0	0	0	5.90	0.1	6.21	795	842	−5.9
11	0	0	0	6.09	0.1	6.25	804	842	−4.7
12	0	0	0	6.12	0.1	6.26	837	842	−0.6
13	0	0	0	5.99	0.0	6.27	769	842	−9.4
14	0	0	0	7.26	0.1	6.27	932	842	9.7
15	0	0	0	7.02	0.0	6.19	853	842	1.3
16	−1	0	0	7.11	0.0	6.46	740	701	5.2
17	1	0	0	6.95	0.0	6.21	925	982	−6.1
18	0	−1	0	7.56	0.0	6.36	900	842	6.5
19	0	1	0	6.87	0.0	6.34	827	842	−1.8
20	0	0	−1	5.11	0.0	7.00	520	622	−19.5
21	0	0	1	7.16	0.9	6.05	734	825	−12.5

[a] Experiments were performed at 37°C until Abs_{ind} 0.8–0.9, when IPTG was added (0.1 mM) for expression of rPsaA for 16 h at 25°C in shake flasks (200 rpm) at an initial pH of 7

[b] Model obtained for rPsaA (mg/L)

[c] Relative error (%) = ((experimental data − predicted by model)/experimental data) × 100 for rPsaA (mg/L)

recombinant mature PsaA was obtained in a soluble form in Tris 20 mM/EDTA 1 mM (pH 8). *E. coli* BL21 Star™ (DE3) without plasmid (negative control) did not show a protein band corresponding to rPsaA. Results for cell growth, residual glucose, pH after 16 h induction, and mature rPsaA (mg/L) are shown in Table 2, and estimated effects of each variable and p values in Table 3.

Cell growth ranged from 4.5 for the poorer media from run 1 (yeast extract concentration equivalent to LB medium without tryptone and with 1 g/L glucose) to 7–7.5 in the higher glucose concentrations. The replicates at the central point had an average Abs_{600nm} of 6.5 ± 0.6 (relative error around 10%). When bacterial growth was compared with 1 g/L and 10 g/L glucose but keeping the same yeast extract and tryptone conditions, a lower growth was obtained at the lower glucose concentration. Cell growth was statistically influenced by the glucose concentration used in the culture medium, whose p value was lower than 0.05.

Glucose was added to the culture medium to repress the T7 promoter (preventing expression before the addition of IPTG) and also as a carbon source for bacterial growth. In the assays performed with 10 g/L, the highest glucose value (runs 5–8), a residual glucose concentration of around 3 g/L was detected after 16 h induction (in the range of 2.3–4 g/L) for all the yeast extract and tryptone conditions, except for run 21 (14.3 g/L yeast extract, 5 g/L tryptone, 10 g/L glucose), for which nearly 1 g/L glucose was detected after 16 h induction (Table 2). The glucose was nearly depleted at the end of the entire process in all the experiments with the lower concentrations (1 and 5.5 g/L). The initial glucose concentration in the culture medium had a statistically significant effect ($p < 0.05$) on residual glucose after 16 h induction (Table 3). When the tryptone concentration corresponded to the −1 level (without any tryptone), glucose uptake was near 100% for the whole range of yeast extract concentrations for 1 and 5.5 g/L initial glucose. Tryptone concentration in the culture medium had no statistical effect on glucose consumption ($p > 0.05$). The pET28a manufacturer recommends the use of 1% (10 g/L) glucose to ensure repression of the

Table 3 Estimated effects, standard errors, and p values for cell growth, rPsaA, residual glucose, and pH after 16 h induction using a face-centered central composite design for evaluation of the effects of yeast extract, tryptone, and glucose concentrations on media formulation

Factor	Cell growth (Abs_{600nm})		Residual glucose (g/L)		Final pH		rPsaA expression (mg/L)	
	Estimated effects[a]	p values[b]	Estimated effects[a]	p values[b]	Estimated effects[a]	p values[b]	Estimated effects[a]	p values[b]
Mean/Interc.	**6.73 ± 0.22**	**0.000**	−0.1 ± 0.2	0.616	**6.26 ± 0.03**	**0.000**	**825 ± 23**	**0.000**
Yeast extract (L) x_1	0.58 ± 0.43	0.206	−0.5 ± 0.4	0.222	**−0.15 ± 0.05**	**0.016**	**280 ± 45**	**0.000**
Yeast extract (Q) x_1^2	−0.10 ± 0.82	0.909	0.8 ± 0.7	0.251	0.05 ± 0.10	0.607	61 ± 85	0.489
Tryptone (L) x_2	0.14 ± 0.43	0.748	0.2 ± 0.4	0.549	0.08 ± 0.05	0.172	−16 ± 45	0.728
Tryptone (Q) x_2^2	0.27 ± 0.82	0.745	0.8 ± 0.7	0.251	0.08 ± 0.10	0.423	123 ± 85	0.177
Glucose (L) x_3	**1.52 ± 0.43**	**0.005**	**2.8 ± 0.4**	**0.000**	**−0.70 ± 0.05**	**0.000**	**204 ± 45**	**0.001**
Glucose (Q) x_3^2	**−1.89 ± 0.82**	**0.042**	**1.7 ± 0.7**	**0.027**	**0.43 ± 0.10**	**0.001**	**−350 ± 85**	**0.002**
x_1 x_2	−0.48 ± 0.48	0.341	0.2 ± 0.4	0.668	−0.03 ± 0.06	0.654	**−93 ± 50**	**0.091**
x_1 x_3	0.37 ± 0.48	0.465	−0.6 ± 0.4	0.176	**−0.17 ± 0.06**	**0.015**	**76 ± 50**	**0.156**
x_2 x_3	−0.38 ± 0.48	0.447	0.3 ± 0.4	0.503	−0.003 ± 0.06	0.967	**−76 ± 50**	**0.157**

[a] Estimated effects ± standard errors

[b] Significance level of the variables and their interactions: $p < 0.05$ indicates that the term is statistically significant (highlighted in bold). For rPsaA expression, the statistical analysis was performed with $p \leq 0.15$

T7 promoter. Results for 1 g/L (tenfold lower than the suggested concentration) confirmed that there was no expression leakage at the lowest glucose concentration [14]. Basal expression is generally undesirable because of the reduction in the available energy for growth during recombinant expression, and it may also lead to inhibition or even cell death if the protein is toxic to the host [18]. Decreasing the glucose concentration did not reduce bacterial growth in this rich medium, i.e., the media contained a high enough yeast extract concentration to ensure a source of carbon and nitrogen.

No significant reduction in the pH was observed in any of the conditions tested owing to the presence of potassium salts. The lowest values were around 6 in the experiments performed with the highest glucose concentrations. The errors associated with the pH measurements calculated using the replicates at the central point were below 1%. Glucose and yeast extract had a statistical influence on the culture's final pH ($p < 0.05$, as shown in Table 3), although this acidification was mitigated by the potassium salts in the medium formulation. As discussed by Manderson et al. [18], glucose is metabolized to acetic acid and this can affect culture growth, e.g., by affecting the pH. The culture medium was also supplemented with glycerol, whose concentration stayed constant at 0.4%. Glycerol is an alternative carbon source to glucose because it does not lead to acetate formation. Acetate accumulation in glucose-bearing media has been described when *E. coli* is grown under anaerobic/oxygen-limiting conditions or in excess glucose, even under aerobic conditions [17].

Mature rPsaA was expressed over 16 h, at 25°C, at a range of 500–1,000 mg/L (Table 2). Replicates at the central point resulted in an average rPsaA concentration of 838 ± 55 mg/L (relative error has been found to be around 10%). Statistical analysis indicated that both the yeast extract and the glucose concentrations had an influence on rPsaA expression in the soluble fraction (Table 3). The main effect of the yeast extract on expressed recombinant PsaA was the linear parameter. The glucose concentration had a significant linear (positive) and quadratic effects on the expression of recombinant mature PsaA. The interactions between all the variables were also statistically significant, considering p values no greater than 0.15.

In order to verify whether the expression of rPsaA was proportional to cell growth, a linear correlation was evaluated using the experimental design results. The linear correlation coefficient obtained was quite low ($R^2 = 0.6$), indicating that the concentration of rPsaA produced was not proportional to cell growth and also depended on protein production per cell. The proportionality of process productivity to the final cell mass and the specific protein productivity is discussed in the literature, as the majority of heterologous proteins are intracellularly accumulated in recombinant *E. coli* [17]. However, as observed by Nikerel et al. [20], the production of a recombinant protein may not have a linear correlation with cell growth. The proportionality between protein expression and cell growth was also evaluated for the different yeast extract concentrations tested in the experimental design (respectively the concentrations equivalent to the LB and TB media) and higher linear correlation coefficients were obtained in these conditions (R^2 near 0.95). The protein production per cell for the LB media (with and without tryptone) was around 25% lower than it was for TB, for both 0.1 and 1% glucose,

confirming the influence of yeast extract concentration on recombinant protein expression ($p < 0.05$), as shown in Table 3.

RSM and ANOVA validation of the model

A model for rPsaA expression was obtained by RSM based on face-centered central composite design in order to select the conditions to optimize protein production. The model is represented in the following equation and graphically in Fig. 1 ($p \leq 0.15$ for statistically significant variables):

$$\begin{aligned} \text{mg rPsaA/L} &= 825 + 140\,x_1 + 102\,x_3 - 175\,x_3^2 \\ &\quad - 46\,x_1x_2 + 38\,x_1x_3 - 38\,x_2x_3. \end{aligned}$$

R^2 was found to be around 0.85, which means that the models could explain 85% of the total variations in the experimental data. The model was also validated by ANOVA. The F value calculated for the model was 12.8, which was higher than the tabulated value $F_{\text{df Reg, df Res, } \alpha} = F_{6,\,14,\,0.15} = 1.91$, where df $_{\text{Reg}}$ and df $_{\text{Res}}$ are the degrees of freedom for the model/regression and the residues, respectively, at the 0.15 probability level (α value). In other words, at the 85% confidence level, $F_{\text{calculated}}$ greater than $F_{\text{tabulated}}$ indicated the significance of the model. The value of R^2 and ANOVA indicated that the second-order polynomial model was capable of representing the experimental data. The model for rPsaA expression (mg/L) was also validated by comparing the experimental and predicted data (as shown in Table 2). The relative errors for each experiment confirmed the model was good enough to represent the experimental data, considering the variability inherent to the complex process under analysis.

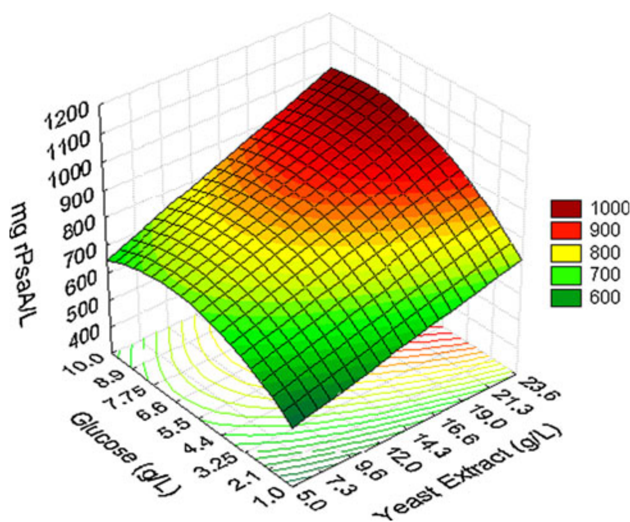

Fig. 1 Response surface for rPsaA expression (mg/L) for glucose and yeast extract without tryptone (at level −1)

Model analysis

As indicated by the estimated effects (Table 3), tryptone was not found to have a significant effect on any of the analyzed responses (cell growth, expressed rPsaA, glucose concentration, pH after 16 h induction), which meant this variable could be taken out of the model because it was tested in the range from 0 to 10 g/L. These results indicated that this component could be removed from culture medium formulation without impairing the process performance. Then, the response surface was plotted (Fig. 1) for tryptone coded as −1 (without this component).

The surface plot in Fig. 1 shows that in the whole range tested, from 5 to 23.6 g/L, the expression of rPsaA was enhanced at higher yeast extract concentrations. Results for the yeast extract concentrations higher than the intermediate condition (~15 g/L) were comparable with the highest values (800–1,000 mg/L) obtained in shake flasks for mature rPsaA expression in TB medium containing 23.6 g/L yeast extract [14]. For the lowest yeast extract values, used with LB medium (5 g/L), a significant expression reduction was verified (around 40–50%), resulting in about 400–600 mg/L. The glucose concentration of 1 g/L resulted in significantly lower expression yields. Recombinant PsaA production was enhanced for higher glucose concentrations until a maximum value near the central point condition. For media formulations without tryptone (Fig. 1), there was a plateau in the mg/L rPsaA response for glucose concentrations higher than 5.5 g/L (at the central point) until the maximum tested concentration (10 g/L), meaning that the rPsaA expression results were similar throughout this glucose range. At the plateau, the maximum rPsaA expression levels were obtained for each yeast extract concentration. At each different glucose and yeast extract concentration, the surface presented a different color as a result of interaction between both variables. At the intermediate yeast extract concentration (~15 g/L), in the plateau observed from 5 to 10 g/L glucose, the result was similar because of the quadratic effect of glucose on rPsaA expression.

Although tryptone is rich in amino acids, salts, and phosphates [23], it was possible to remove it from the culture medium because the carbon and nitrogen sources from the yeast extract and glucose were enough for cell growth and recombinant protein expression in the experiments performed in shake flasks. In high density cultivations of *E. coli* in a bioreactor, obtaining higher growth rates, these concentrations would have to be adjusted to prevent substrate limitation. For other proteins described in the literature [23], and pneumolysin from *S. pneumoniae* expressed in the same system as PsaA [14], tryptone had a positive effect on protein production in *E. coli*. The enhancement of recombinant protein expression in *E. coli*

with a higher yeast extract concentration was also described in other works using similar shake flask conditions and in the same range of concentrations in the culture medium [4, 29, 30]. Chen et al. [4] reported that yeast extract was effective as a nitrogen source to improve recombinant protein expression.

Validation of experimental design results for medium optimization

Statistical and model analyses were used to define an optimal medium composition for mature rPsaA expression at 16 h induction with 0.1 mM IPTG at 25°C. Similar protein expression results for the culture medium with and without tryptone led to the removal of this component from the optimized medium. Yeast extract concentration should be kept to a minimum while keeping the highest possible levels of rPsaA expression; its optimization was defined around the intermediate condition (15 g/L), where the highest values (near to 1,000 mg/L) were obtained with a lower yeast extract concentration than in the TB medium, as discussed above. The optimal glucose concentration was chosen in view of the fact that no expression leakage was verified in the range of glucose concentrations tested (0.1–1%), and to ensure growth without carbon source limitations in view of the removal of tryptone from the medium and the reduction of yeast extract. As the cost of glucose is low, there is less batch-to-batch variation, and the pH reduction associated with its metabolism did not have a significant influence (and drastic acidification is prevented by the presence of potassium salts), 0.8% (8 g/L) was the glucose concentration selected for use in the optimal culture medium in shake flasks.

The results for cell growth and mature rPsaA expression after 16 h induction with 0.1 mM IPTG at 25°C, 200 rpm, in 500-mL shake flasks, and 100 mL growth medium (15 g/L yeast extract, 9.4 g K_2HPO_4, 2.2 g KH_2PO_4, 8 g/L glucose supplemented with 50 μg/mL kanamycin and 0.4% glycerol) were in the same range as the highest values obtained in the experimental design, but achieved at a reduced cost in comparison to the TB medium [14]. Duplicate runs were performed for 16 h growth of seed culture and average results were Abs_{600nm} 7.0, residual glucose 1.5 g/L, pH 6.1, and 931 mg rPsaA/L. The experimental results for rPsaA (mg/L) agreed well with those predicted by the model in optimized medium conditions (Table 4). The error verified for the model was low in the conditions near the maximum values and the validation showed the objectives were attained.

The entire process used in the experimental design experiments for media optimization and validation took around 40 h: 16 h for overnight seed growth until saturation (Abs_{600nm} near 5); 4–5 h for storage of seed culture at 4°C; 2 h growth until Abs_{ind} 0.8–0.9 at 37°C; 16 h for overnight rPsaA expression at 25°C. Cold storage was used to adjust the interval for both overnight processes; however, it could be eliminated if seed growth was performed for 7 h, until the exponential phase, instead of 16 h. The performance of the process under the validation condition using 7 h seed culture growth was similar to 16 h seed growth.

Evaluation of the effects of salt and seed conditions

On the basis of the results of the seed condition adjustment for the validation of the experimental design for medium optimization, the influence of this variable was analyzed. Sunitha et al. [27] evaluated the effect of the growth time of seed used as an inoculum on experimental design, with around 3 h seed age leading to enhanced recombinant protein expression in comparison with overnight seed culture. We also tested the substitution of the potassium salts in the TB medium (9.4 g/L K_2HPO_4 and 2.2 g/L KH_2PO_4) for NaCl (salt present in LB), aiming to reduce costs and enhance the purification performance. A central composite design was used to evaluate the influence of seed growth (7 and 16 h) and NaCl concentration in the medium (0, 5 and 10 g/L), using duplicates of each condition. The results for the central composite experimental design for the two variables are shown in Table 5.

In the medium with potassium salts, the pH fell less (final pH around 6) than when NaCl was used (Table 5) in the medium composition (final pH in a range of 5–6). However, NaCl can replace potassium salts, because pH

Table 4 Experimental results for rPsaA expression (mg/L) compared with results predicted by the model under optimized medium conditions

Parameter	Range studied (g/L)	Optimal values	Coded values		Predicted	Experimental[a]
Yeast extract	5–23.6	15 g/L	$x_1 = 0.075$	rPsaA (mg/L)	864	931
Tryptone	0–10	Without tryptone	$x_2 = -1$		Relative error = 7.2%	
Glucose	0.1–10	8 g/L	$x_3 = 0.555$			

Optimized conditions were 15 g/L yeast extract, 8 g/L glucose, potassium salts (9.4 g K_2HPO_4, 2.2 g KH_2PO_4). Seed growth was conducted for 16 h until saturation

[a] Experimental values are the means of the duplicates (error around 10%)

Table 5 Results for different conditions of central composite experimental design for two variables: seed age and NaCl concentration at 16 h induction

Run[a]	Exp[b]	Seed age (h)[c]	NaCl (g/L)	Cell growth (Abs_{600nm})	Residual glucose (g/L)	Final pH	rPsaA (mg/L)
1	1	7 (−1)	0 (−1)	7.29	4.6	5.32	1,031
2	1	7 (−1)	0 (−1)	6.21	4.0	5.40	732
3	2	16 (+1)	0 (−1)	6.69	4.3	5.38	800
4	2	16 (+1)	0 (−1)	5.90	4.1	5.44	755
5	3	7 (−1)	10 (+1)	6.16	5.1	5.35	824
6	3	7 (−1)	10 (+1)	5.69	4.2	5.43	773
7	4	16 (+1)	10 (+1)	4.75	5.1	5.18	735
8	4	16 (+1)	10 (+1)	5.21	4.1	6.02	731
9	CP1	7 (−1)	5 (0)	7.38	3.9	5.19	928
10	CP1	7 (−1)	5 (0)	7.72	4.0	5.14	1,017
11	CP2	16 (+1)	5 (0)	7.76	4.4	5.64	939
12	CP2	16 (+1)	5 (0)	7.86	4.5	5.76	1,049

Variables were determined in a medium with 15 g/L yeast extract, 8 g/L glucose supplemented with 50 µg/mL kanamycin and 0.4% glycerol, pH 7

[a] Experiments were performed at 37°C until induction (Abs_{ind} 0.8–0.9) with 0.1 mM IPTG for 16 h at 25°C, 200 rpm, in 500 mL shake flasks

[b] Experiments performed in duplicate

[c] Validated medium tested with 16 h seed growth (until saturation) and 7 h seed growth (until the exponential phase)

reduction did not significantly influence the yield of expressed protein, as indicated by the results shown in Table 5. The residual glucose concentrations after 16 h induction without any salt and with NaCl (4–5.1 g/L, Table 5) were more than three times higher than the residual glucose when potassium salts were used (1.5 g/L). These results show the influence of the medium composition, such as ion concentrations, on bacterial metabolism. Reducing glucose levels in culture media with NaCl could be considered for future studies, which would reduce costs and curb acidification.

Neither variable was statistically significant for cell growth, rPsaA expression, residual glucose, or pH after 16 h induction under the different seed culture and NaCl concentrations listed in Table 5, because the *p* values associated with both parameters' effects were higher than 0.05, indicating that all the conditions resulted in similar process performances. However, the salt present in the medium influences the purification of rPsaA by ion exchange. Previous studies found that the addition of NaCl reduced the interaction of the contaminants in the column, enhancing yields and purities obtained for mature rPsaA purification in the weak anionic Sepharose HiTrap DEAE FF [14]. Thus, the optimized condition was chosen that had the shorter process time (seed culture for 7 h growth) and that enhanced the yield purification process by the presence of NaCl in the medium for cell growth (using a salt concentration of 5 g/L NaCl). The growth rate at the exponential phase obtained for the seed culture of *E. coli* BL21 Star™ (DE3)/pET28a/*psaA* for 7 h at 37°C in TB was 1.15 h^{-1}, a similar value to growth rates associated with plasmid-free, wild-type *E. coli* or μ before induction, indicating that the recombinant strain was at its best state under these conditions [12].

The culture medium formulation and seed conditions were optimized and the process was performed in 25 h as follows: 7 h seed culture growth in TB until the exponential phase was reached (around Abs_{600nm} 2.5–3); this culture was used as the seed for 2 h growth at 37°C in the optimized medium (15 g/L yeast extract, 5 g/L NaCl, 8 g/L glucose) until Abs_{ind} 0.8–0.9, when 0.1 mM IPTG was added for overnight rPsaA expression for 16 h at 25°C. Under these conditions around 1 g/L soluble recombinant protein was obtained (condition CP1 in Table 5).

The entire process under the optimized seed and medium conditions took 25 h, which means around 35% time saving in comparison to the 40 h of the previous process [14]. These results allowed time and process cost savings, but kept similar expression levels, around 1 g mature rPsaA/L per culture medium, corresponding to yields as high as those of soluble recombinant protein in *E. coli* described in the literature [5]. In terms of process productivity, this expression level means 40 (mg/L)/h, i.e., a 100% increase in comparison to the 20 (mg/L)/h obtained in the expression in TB [14].

Confirmation of optimized seed and culture medium conditions in 400 mL scale-up

The optimized medium (15 g/L yeast extract, 5 g/L NaCl, 8 g/L glucose supplemented with 0.4% glycerol and

50 μg/mL kanamycin) and seed conditions (growth for 7 h, until the exponential phase) were performed in triplicate using a 400-mL volume in 2-L shake flasks. Under these conditions, cell growth (5.1 ± 0.4) was around 20% lower than the experiments performed using 100 mL medium, leading to 850 ± 50 mg rPsaA/L obtained in the soluble fraction. The yield obtained from 400 mL was 12.5% lower than that obtained in the experiments using 100 mL culture medium in 500-mL shake flasks. This decrease can be associated with a fall in oxygen transfer, because despite keeping the same ratio of culture and shake flask volumes in both conditions, aeration is not so efficient in higher volumes [13]. A similar reduction in expression in the 400-mL culture/2-L shake flask was obtained for the TB medium (Abs_{600nm} around 6.0 and 851 mg rPsaA/L). Glucose after 16 h induction (5.3 ± 0.9 g/L) and pH (5.5) in 400 mL were in the same range as the experiments using 100 mL culture medium (Table 5), but slightly higher due to the reduced cell growth.

Conclusions

Experimental design techniques proved to be valuable tools for the optimization of process conditions for the expression of soluble mature rPsaA in *E. coli* BL21 Star^{TM} (DE3)/pET28a. The sequence of two-level experimental designs employed made it possible to evaluate the effects of the medium composition and seed growth in order to enhance the production of this potential vaccine candidate, achieving higher process yields and productivities (by reducing process time) and lower raw material costs. In all conditions in which the expression was performed, the mature rPsaA protein was retained in the soluble fraction. Tryptone could be removed altogether, which fulfills health legislation restrictions, leading to the formulation of an animal-derived material-free culture medium. Yeast extract, glucose, and NaCl concentrations (rather than more expensive potassium salts) were optimized at their intermediate concentrations, resulting in a culture medium comprised of 15 g/L yeast extract, 8 g/L glucose, and 5 g/L NaCl, leading to a significant cost reduction (to about a third of the original value) in comparison to the TB medium used formerly for expression of this antigen in *E. coli* [14], and yielding around 1,000 mg/L rPsaA. Instead of growing the seed culture for 16 h (until saturation), the process can be conducted with 7 h seed growth until the exponential phase. These results allowed a 35% reduction of the entire process time and a 100% enhancement of the process productivity in comparison to the previous process (40 (mg/L)/h cf. 20 (mg/L)/h obtained in the expression of rPsaA in TB medium).

In addition, the development of chemically defined media could be a feasible alternative for substituting the complex components used in culture media, especially the inexpensive complex carbon and nitrogen sources that are mostly used to yield high productivities in commercial bioreactions [9, 19]. Fontani et al. [9] showed that chemically defined media are attractive for the production of health products from a regulatory point of view and also ensure higher process production consistency (avoiding batch-to-batch variability). A suitable approach for developing the formulation of this kind of media with large numbers of process variables with an experimental workload as small as possible is to conduct batch experiments in a parallel approach applying experimental design tools and then to perform scaling-up to bioreactors adjusting aeration, agitation, and substrate concentrations to prevent substrate limitation due to the higher growth rates reached in high density cultivations of *E. coli*.

Acknowledgments Bio-Manguinhos, PDTIS (Programa de Desenvolvimento Tecnológico de Insumos para Saúde) and PAPES V (Programa Estratégico de Apoio à Pesquisa em Saúde) from Fundação Oswaldo Cruz (Fiocruz) supported this work. We thank Sinéa Mendes de Andrade MSc, Cláudia Maria da Conceição MSc, and Dr. Filipe Soares Quirino da Silva (INCQS/Fiocruz) for the use of the densitometer, Dr. Ana Carolina Andrade de Góes (Bio-Manguinhos/Fiocruz) for help in purification analysis, and Prof. Dr. Tito Lívio Moitinho Alves (COPPE/UFRJ) for fruitful discussions.

References

1. Bogaert D, Hermans PWM, Adrian PV, Rümke HC, de Groot R (2004) Pneumococcal vaccines: an update on current strategies. Vaccine 22:2209–2220
2. Briles DE (2004) Protection of the elderly from pneumococcal pneumonia with a protein-based vaccine? Mech Ageing Dev 125:129–131
3. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, Virolainen A, Swiatlo E, Hollingshead S (2000) Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 68:796–800
4. Chen Y, Xing X-H, Ye F, Kuang Y, Luo M (2007) Production of MBP-HepA fusion protein in recombinant *Escherichia coli* by optimization of culture medium. Biochem Eng J 34:114–121
5. Choi JH, Keum KC, Lee SY (2006) Production of recombinant proteins by high cell density culture of *Escherichia coli*. Chem Eng Sci 61:876–885
6. Choi WC, Oh BC, Kim HK, Lee ES, Oh TK (2002) Medium optimization for phytase production by recombinant *Escherichia coli* using statistical experimental design. J Microbiol Biotechnol 12:490–496
7. De BK, Sampson JS, Ades EW, Huebner RC, Jue DL, Johnson SE, Espina M, Stinson AR, Briles DE, Carlone GM (2000) Purification and characterization of *Streptococcus pneumoniae* palmitoylated pneumococcal surface adhesin A expressed in *Escherichia coli*. Vaccine 18:1811–1821
8. Douce G, Ross K, Cowan G, Ma J, Mitchell TJ (2010) Novel mucosal vaccines generated by genetic conjugation of

heterologous proteins to pneumolysin (PLY) from *Streptococcus pneumoniae*. Vaccine 28:3231–3237
9. Fontani S, Niccolai A, Kapat A, Olivieri R (2003) Studies on the maximization of recombinant *Helicobacter pylori* neutrophil-activating protein production in *Escherichia coli*: application of Taguchi robust design and response surface methodology for process optimization. World J Microbiol Biotechnol 19:711–717
10. Gor DO, Ding X, Li Q, Schreiber JR, Dubinsky M, Greenspan NS (2002) Enhanced immunogenicity of pneumococcal surface adhesin A by genetic fusion to cytokines and evaluation of protective immunity in mice. Infect Immun 70:5589–5595
11. Hao DC, Zhu PH, Yang SL, Yang L (2006) Optimization of recombinant cytochrome P450 2C9 protein production in *Escherichia coli* DH5α by statistically-based experimental design. World J Microbiol Biotechnol 22:1169–1176
12. Ihssen J, Kowarik M, Dilettoso S, Tanner C, Wacker M, Thöny-Meyer L (2010) Production of glycoprotein vaccines in *Escherichia coli*. Microb Cell Fact 9:61–73
13. Islam RS, Tisi D, Levy MS, Lye GJ (2007) Framework for the rapid optimization of soluble protein expression in *Escherichia coli* combining microscale experiments and statistical experimental design. Biotechnol Prog 23:785–793
14. Larentis AL, Argondizzo APC, Esteves GS, Jessouron E, Galler R, Medeiros MA (2011) Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage. Protein Expr Purif 78:38–47
15. Lavall CB, Andrade ALSS, Pimenta FC, Andrade JG, Oliveira RM, Silva SA, Lima EC, Di Fabio JL, Casagrande ST, Brandileone MCC (2006) Serotypes of carriage and invasive isolates of *Streptococcus pneumoniae* in Brazilian children in the era of pneumococcal vaccines. Clin Microbiol Infect 12:50–55
16. Lee KM, Rhee CH, Kang CK, Kim JH (2006) Sequential and simultaneous statistical optimization by dynamic design of experiment for peptide overexpression in recombinant *Escherichia coli*. Appl Biochem Biotechnol 135:59–80
17. Lee SY (1996) High cell-density culture of *Escherichia coli*. Trends Biotechnol 14:98–105
18. Manderson D, Dempster R, Chisti Y (2006) A recombinant vaccine against hydatidosis: production of the antigen in *Escherichia coli*. J Ind Microbiol Biotechnol 33:173–182
19. Niccolai A, Fontani S, Kapat A, Olivieri R (2003) Maximization of recombinant *Helicobacter pylori* neutrophil activating protein production in *Escherichia coli*: improvement of a chemically defined medium using response surface methodology. FEMS Microbiol Lett 221:257–262
20. Nikerel İE, Öner E, Kirdar B, Yildirim R (2006) Optimization of medium composition for biomass production of recombinant *Escherichia coli* cells using response surface methodology. Biochem Eng J 32:1–6
21. Pan H, Xie Z, Bao W, Zhang J (2008) Optimization of culture conditions to enhance *cis*-epoxysuccinate hydrolase production in *Escherichia coli* by response surface methodology. Biochem Eng J 42:133–138
22. Pimenta FC, Miyaji EN, Arêas APM, Oliveira MLS, de Andrade ALSS, Ho PL, Hollingshead SK, Leite LCC (2006) Intranasal immunization with the cholera toxin B subunit-pneumococcal surface antigen A fusion protein induces protection against colonization with *Streptococcus pneumoniae* and has negligible impact on the nasopharyngeal and oral microbiota of mice. Infect Immun 74:4939–4944
23. Pistorino M, Pfeifer BA (2009) Efficient experimental design and micro-scale medium enhancement of 6-deoxyerythronolide B production through *Escherichia coli*. Biotechnol Prog 25:1364–1371
24. Rajam G, Anderton JM, Carlone GM, Sampson JS, Ades EW (2008) Pneumococcal surface adhesin A (PsaA): a review. Crit Rev Microbiol 34:131–142
25. Rodrigues MI, Iemma AF (2009) Planejamento de experimentos e otimização de processos, 2nd edn. Casa do Pão Editora, Campinas
26. Seo JY, Seong SY, Ahn BY, Kwon IC, Chung H, Jeong SY (2002) Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin A encapsulated in microspheres. Infect Immun 70:1143–1149
27. Sunitha K, Kim Y-O, Lee J-K, Oh T-K (2000) Statistical optimization of seed and induction conditions to enhance phytase production by recombinant *Escherichia coli*. Biochem Eng J 5:51–56
28. Tai SS (2006) *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. Crit Rev Microbiol 32:139–153
29. Volontè F, Marinelli F, Gastaldo L, Sacchi S, Pilone MS, Pollegioni L, Molla G (2008) Optimization of glutaryl-7-aminocephalosporanic acid acylase expression in *E. coli*. Protein Expr Purif 61:131–137
30. Zhao J, Wang Y, Chu J, Zhang S, Zhuang Y, Yuan Z (2008) Statistical optimization of medium for the production of pyruvate oxidase by the recombinant *Escherichia coli*. J Ind Microbiol Biotechnol 35:257–262