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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

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Summary

The neutrophil-activating protein of *Helicobacter pylori* (HP-NAP) is a major antigen responsible for the generation of immune response in an infected individual. The cloning and expression of the gene corresponding to neutrophil-activating protein (NAP) were followed by process development for enhanced production and purification. The production process was developed in two parts. In the first part, some of the cultivation medium components (viz. carbon to nitrogen ratio, concentrations of sodium polyphosphate and magnesium sulphate) were optimized using the Taguchi robust experimental design. The intracellular NAP production level after 24 h of cultivation was considered as the target function or the dependent variable. There was a 76.8% increase in the NAP production level. Using this optimal medium composition obtained in the first part, the temperature of cultivation and the pH of cultivation medium were optimized in the second part. The NAP production level at hour 30 of cultivation was considered as the target function or the dependent variable. The optimal values for these two independent variables were 37.2 °C and 6.3 respectively. At this combination of temperature and pH, the theoretical maximum NAP production level was 1280 mg l^{-1}. This optimal combination was verified experimentally and the NAP production level was found to be 1261 mg l^{-1}. The optimization of the cultivation conditions resulted in a 61.5% increase in NAP production level. About a 2.91-fold overall increase in NAP production level at hour 24 of cultivation was achieved through process optimization.

Introduction

The human pathogen *Helicobacter pylori* is the causative agent of active chronic gastritis (Solnik & Tompkins 1993). The infection by *H. pylori* is strongly associated with the development of gastric and duodenal ulcers (Blaser & Parsonnet 1994). *H. pylori* or cell-bound compounds released from the bacterium can cause chemotaxis (Craig *et al.* 1992), activate neutrophils to strong oxidative burst (Rautelin *et al.* 1993) and induce an increased adhesion of neutrophils to endothelial cells (Yoshida *et al.* 1993). A neutrophil-activating protein (NAP) from *H. pylori* has been identified (Evans *et al.* 1995). This 150 kDa protein was isolated from aqueous extracts of the bacterium. The NAP induces an increased expression of CD11b/CD18 on neutrophils and promotes the adhesion of neutrophils to endothelial cells. This protein was thought to be a good immunogen for development of a vaccine against *Helicobacter pylori.*

Since cultivation of *H. pylori in vitro* was shown to be difficult and time-consuming (Olivieri *et al.* 1993), the *H. pylori*-NAP (HP-NAP) gene was identified and cloned into *Escherichia coli*. It was expressed as a soluble constitutive protein (Petracca R. & Galli G.; IRIS, Chiron S.r.L., Siena, Italy, unpublished report). It was found that mice immunized with a recombinant, urea-solubilized NAP fused to *E. coli* thioredoxin were protected against *H. pylori* infection (Petracca R. & Galli G.; IRIS, Chiron S.r.L., Siena, Italy, unpublished report). This finding further confirmed that HP-NAP was an immunogen which could possibly be used for vaccine development.

Emphasis has therefore been given to a process development for enhanced production of NAP using

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chemically defined medium. A detailed study on the improvement of such a medium (containing sodium polyphosphate as the only phosphate source) was conducted in the present laboratory using response surface methodology (RSM) which resulted in a substantial increase in NAP production level (unpublished report). However, when the batch of sodium polyphosphate was changed, a drastic decrease of cell growth as well as NAP production level was observed. A new optimization program was then designed to circumvent this problem and to increase the NAP production level. A systematic study was conducted where both chemical and physical parameters governing the cultivation process were examined for their possible effect on the microbial physiology as well as on intracellular NAP production level.

A rapid method of optimization with orthogonal arrays (Taguchi 1987; Logothetis & Wynn 1990) was thought to be useful in order to optimize the chemical parameters viz. the medium components in a minimum number of experimental trials. This method is simple, involving a system of tabulated designs (arrays) that allow for the maximum number of main effects to be estimated in an unbiased (orthogonal) manner with a minimum number of runs in the experiment. It also attempts to maximize signal-to-noise ratio (SNR). The modelling that is done essentially relates SNR to the control variables in a 'main effect only' approach.

Since both temperature of cultivation and the pH of the culture medium affect the growth of the micro-organism and production of recombinant protein, a detailed study was carried out in order to find an optimal condition for these two parameters. RSM was used for exploring a predefined experimental domain and to find both the individual and combined effects of pH and temperature on recombinant NAP production.

Materials and methods

Plasmid and micro-organism

The HP-NAP gene was amplified by PCR using pTrxFus-NAP as template and inserted in to *Escherichia coli*/*Bacillus subtilis* shuttle expression vector pSM214 (Petracca R. & Galli G.; IRIS, Chiron S.r.L. Siena, Italy, unpublished report). The chloramphenicol resistance gene was used as the selection marker. The plasmid was used for the transformation of *E. coli* MM294-1 which was an auxotroph for thiamine. The recombinant organism was named as *E. coli* MM294-1 NAP. It was obtained from the Chiron S.r.L. bacterial culture collection. Working seed was prepared by growing this organism in complex medium [per litre of deionized water: glucose, 5.0 g; yeast extract (Difco), 5.0 g; yeast extract (PTK, Deutsche Hefewerke GmbH, Germany), 10.0 g; NaCl, 8.0 g and chloramphenicol, 20.0 mg] till the optical density (OD) reached 0.4. The culture was stored as 300 μl aliquots in 10% glycerol (vv^{-1}) at −70 °C.

Media and culture conditions

The medium for inoculum contained (per litre of deionized water): glycerol, 20 g; KH_2PO_4, 13.5 g; $(NH_4)_2HPO_4$, 4.0 g; $MgSO_4 \cdot 7H_2O$, 1.4 g; citric acid, 1.7 g; thiamine 10.0 mg; chloramphenicol, 20.0 mg (Lee & Lee 1996). Thirty millilitre of this medium was prepared and the pH was adjusted to 7.0 by adding 3 M NaOH. Twenty-five millilitre of this medium was filter sterilized (0.2 μm pore size, Millipore) in a sterile 300 ml conical flask.

The production medium[1] contained (per litre of deionized water): glycerol, 19.66–58.97 g; $(NH_4)_2SO_4$, 7.26 g; sodium polyphosphate $(NaPO_3)_7$ (Budenheim-GmbH, Germany), 4.0–12.0 g; $MgSO_4 \cdot 7H_2O$, 2.0–4.0 g; K_2SO_4, 7.24 g; thiamine 10.0 mg; chloramphenicol, 20.0 mg and antifoam (polypropylene glycol, Aldrich), 500 μl. The concentration of glycerol, sodium polyphosphate and $MgSO_4 \cdot 7H_2O$ were varied according to the experimental design. $(NH_4)_2SO_4$ was dissolved in 990 ml of deionized water in the reactor and autoclaved for 30 min at 121 °C. Glycerol, $(NaPO_3)_7$, $MgSO_4 \cdot 7H_2O$ and K_2SO_4 were dissolved in deionized water separately and then mixed together under constant stirring. This mixture was filter sterilized and pumped into the autoclaved bioreactor after the temperature was brought down to the process temperature (variable). Thiamine and chloramphenicol (dissolved in 50% ethanol) were filter sterilized and were added to the bioreactor with a sterile injection syringe.

The trace element stock solution was composed of the following inorganic compounds: H_2SO_4 (37%), 10 ml; $FeCl_3 \cdot 6H_2O$, 54.0 g; $ZnSO_4 \cdot 7H_2O$, 22.0; $CoCl_2 \cdot 6H_2O$, 0.5 g; $Na_2MoO_4 \cdot 2H_2O$, 0.5 g; $CuSO_4 \cdot 5H_2O$, 0.13 g; boric acid, 0.5 g; $MnSO_4 \cdot H_2O$, 11.0 g; sodium selenite, 0.02 g. The solution was filter sterilized into a sterile bottle and was stored at +4 °C. The diluted solution (1×) was prepared by diluting this 1000× stock solution with sterile deionized water.

For the studies on optimization of temperature of cultivation and pH of the culture medium, the optimal combination of the medium components obtained from the result of Taguchi Robust Design was used.

Working seed of *E. coli*-NAP (250 ml) was inoculated into 25 ml of chemically defined medium contained in a sterile 300 ml conical flask. The flask was incubated in temperature controlled rotary shaker for 15 h at 35 °C and at 150 rev min^{-1}. About 4.5 ml of this culture was inoculated into 1.5 l of chemically defined production medium contained in the bioreactor.

The production of NAP was carried out in 3.0 l batch bioreactor [Applikon Dependable Instruments, The Netherlands; Pyrex glass vessel; diameter (d_t) =

[1] A patent application (P026922WO) has been filed on the basal medium composition.

13.0 cm height (h) = 23.4 cm; $h.d_t^{-1} = 1.8$] with a working volume of 1.5 l. The bioreactor was equipped with two 6-bladed disc turbine impellers [impeller diameter (d_i) = 4.5 cm; $d_i d_t^{-1} = 0.35$, direct stirring by top mounted motor], air sparger, pH probe (combined electrode, Applisens, the Netherlands) and DO probe (Mettler Toledo, Switzerland). pH, dissolved oxygen tension (DOT), agitation and temperature were controlled by a digital control unit (ADI 1030) which was connected to a microcomputer (IBM 286) loaded with BioXpert (version 1.14, Applikon Italia, S.R.L.) process control software. On-line data for pH, temperature and DOT were collected using this software. The agitation was kept constant at 650 rev min^{-1}. The dissolved oxygen level was controlled automatically at 75% of saturation by sparging air or a mixture of air and pure oxygen when necessary. The temperature was maintained at 35 °C with a thermostatic water circulator (Haake, Germany) and the pH was maintained at 7.0 with 3 M NaOH. Foam was suppressed, when necessary, by the addition of antifoam agent (polypropylene glycol, Aldrich). For the studies on the effect of temperature and pH, these two parameters were varied according to the requirement of experimental design. The cultivation in the reactor was carried out for 30 h. Samples were withdrawn at regular intervals and the growth was checked immediately by measuring the OD of the sample at 590 nm. Samples were then stored in sterile tubes at −20 °C for further analysis.

Experimental design

Taguchi's methodology for robust parameter design involves the use of orthogonal designs, where an orthogonal array involving control variables is crossed with an orthogonal array for the noise variables. For example, in a $2^2 \times 2^2$, the 2^2 for the control variables is called the inner array and the 2^2 for the noise variables is called the outer array. The result is a 16 run design called a crossed array. The corners of the inner array represent (−1, −1), (−1, 1), (1, −1) and (1, 1) for the control variables thus constituting the main design. For the optimization of medium components, a 2^3 inner array was chosen with the coordinates described in Table 1.

Table 1. Variables and the coordinates in Taguchi robust design for medium components optimization.

Experiment number	Glycerol[a], g l^{-1} (X_1)	$(NaPO_3)_7$, g l^{-1} (X_2)	$MgSO_4 \cdot 7H_2O$, g l^{-1}, (X_3)
1	19.66 (−1.0)	4.0 (−1.0)	2.0 (−1)
2	19.66 (−1.0)	12.0 (1.0)	4.0 (1.0)
3	58.97 (1.0)	4.0 (−1.0)	4.0 (1.0)
4	58.97 (1.0)	12.0 (1.0)	2.0 (−1.0)

The coded values (coordinates) are given in the parenthesis.
[a] The concentration of glycerol was varied in order to obtain C to N ratio of 5 to 15.

Table 2. Optimization of temperature and pH: independent variables in the 2^2 factorial design.

Coded values	Variable	
	X_1 (temperature, °C)	X_2 (pH)
−1.414	27.95	6.29
−1.0	30	6.5
0	35	7.0
1.0	40	7.5
1.414	42.05	7.7

x_i = coded value of the variable X_i; x_1 = (temperature − 35)/5; x_2 = (pH − 7.0)/0.5.

Introduced by Box & Wilson (1951), central composite design (CCD) is composed from the superimposition of two level factorial design and three level 'star' design.

The dependent variables (factors) were coded according to the following equation:

$$x_i = (X_i - X_0)/\Delta X_i, \tag{1}$$

where, x_i is the dimensionless value of an independent variable, X_i is the real value of that independent variable, X_0 is the real value of that independent variable at the centre point and ΔX_i is the step change.

In this study the temperature of cultivation (X_1) and pH of the culture medium (X_2) were chosen as independent variables. The levels for these variables are given in Table 2.

The following second order quadratic model was used for the description of system behaviour.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 + \varepsilon, \tag{2}$$

where Y represents predicted response, x_i is the coded value of independent variables, β_0 is the interception coefficient, β_i is the linear effect, β_{ii} is the squared effects, β_{ij} is the interaction effect and ε is the random error in Y. The random errors are assumed to be independently distributed as normal variables with a zero mean and a common variance, σ^2.

All statistical calculations and data representations were performed by a combined usage of JMP (SAS Institute Inc., USA), Statistica (Statsoft Inc., USA) and Matlab (Mathwork Inc., USA).

Optimization of the polynomial equation for function maximization

The optimal combination of the variables which resulted in maximum theoretical NAP production level was obtained by optimizing the polynomial equation within the boundary of −1.414 and +1.414. The optimization was done using a subprogram (Optim) embedded in Matlab (Mathworks Inc., USA).

Quantification of NAP by densitometry

The fermentation sample was defrosted at room temperature and was vortexed well. The OD of this sample was normalized to $OD_{590} = 2.0$ by adding appropriate proportions of phosphate buffered saline (PBS) and the original fermentation sample in an Eppendorf tube. One millilitre of this diluted cell sample was centrifuged at 13,000 rev min^{-1} (16110 × *g*) for 10 min and the pellet was re-suspended in 1 ml of fresh PBS. This cycle was repeated twice. This washed sample (25 μl) was then pipetted into a clean Eppendorf tube and was centrifuged at 13,000 rev min^{-1} (16,110 × *g*) for 10 min. The pellet was re-suspended in reducing buffer (1×) containing sodium dodecyl sulphate (SDS) and dithiothreitol (DTT). The suspension was incubated in a boiling water bath for 10 min, cooled down to room temperature and centrifuged for 10 s. The samples were subjected to SDS-PAGE (Laemmli 1970).

The gels were scanned using a scanner (SharpJX300) and the images were stored for further usage in quantification software. The scanning was done at 256 grey scale with 150 dpi resolution.

The image of the gel was imported to Image Master 1D Elite (Version 3.0, Pharmacia). A calibration plot was drawn using the density of the bands for purified NAP (concentrations of which were known). The density of the band in unknown sample was then extrapolated automatically from this calibration plot. The amount of NAP in the sample was calculated using an appropriate conversion equation and was expressed in terms of mg l^{-1}.

Estimation of NAP in purified standard by Micro BCA

The Pierce Micro BCA Protein Assay Reagent (Pierce, US Patent No. 4839295) was used for the estimation of protein in the NAP standard (purified).

Estimation of cellular growth

The cellular growth was monitored by measuring the OD (at 590 nm) with a u.v./vis. spectrophotometer (Novaspec II, Pharmacia-KKB, Sweden). The fermentation sample was diluted with physiological saline (0.9% NaCl) if needed and the absorbance was measured against physiological saline.

Estimation of cell dry weight

Fermentation sample (1 ml) was dispensed into a dry pre-weighed Eppendorf tube and was centrifuged at 13,000 rev min^{-1} (16,110 × *g*) for 10 min. The supernatant was stored for further analysis of metabolite. The pellet was washed with deionized water and then dried at 37 °C till a constant weight was obtained. The cellular growth was expressed in terms of cell dry weight (CDW, g l^{-1}).

Estimation of residual glycerol and acetic acid

Residual glycerol and acetic acid formed were measured by enzymatic analytical kits (Boehringer Mannheim, catalogue nos. 148270 and 148261, respectively).

Results and discussion

The media employed to support high productivities in commercial fermentations are predominantly formulated with inexpensive complex carbon and nitrogen sources. As a result, fermentation performance may vary because of the lot-to-lot variation inherently associated with these ill-defined components. This undesirable variability in productivity has a significantly higher impact on biological products used for therapeutic purposes. One approach for reducing performance variability while maintaining or even enhancing productivity is to replace the complex medium with chemically defined medium which can be used on an industrial scale in order to achieve consistency in the production level and to simplify large scale downstream processing. It also facilitates regulatory compliance with good manufacturing practices since it does not contain any material of biological origin.

One of the most important salient features of the present medium is the complete replacement of common orthophosphate with polyphosphate. In microbial cells, inorganic polyphosphate (polyP) plays a significant role in increasing cell resistance to unfavourable environmental conditions and in regulating different biochemical processes. Intracellular PolyP is a poly-functional compound. The most important of its functions are the following: phosphate and energy reservation, cation sequestration and storage, membrane channel formation, participation in phosphate transport, involvement in cell envelope formation and function, gene activity control, regulation of enzyme activities, and a vital role in stress response and stationary-phase adaptation. In the fermentation perspective, addition of polyphosphate to the medium exhibits marked advantages, viz. polyphosphate costs no more than sodium or potassium orthophosphate, is more soluble than any other inorganic phosphate source, and does not form metallophosphate precipitates when mixed with other nutrients in the proportions found in fermentation media.

Having identified an appropriate starting medium, medium optimization to increase the production level of NAP was investigated employing an alternative optimization strategy which uses very simple robust statistically designed experiments that allow the investigator to evaluate more than one variable at a time. The approach starts with the identification of those variables that have a significant effect on the desired response from a large number of potential variables. The next step is usually to find the combination of these variables that supports the best acceptable response in a timely manner using a response surface design. Thus, statistically designed

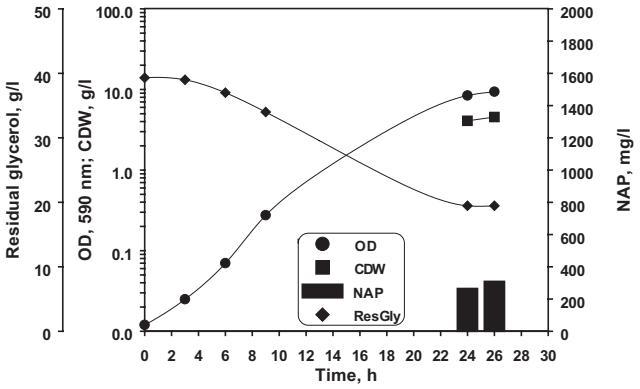

Figure 1. Time profiles of recombinant *E. coli*-NAP cultivation in unoptimized chemically defined medium.

experiments are effective because they supply the needed information, and efficient because they expend a minimum of resources. On the basis of previous knowledge on microbial physiology, carbon to nitrogen ratio, concentrations of Na-polyphosphate and magnesium sulphate were chosen as the independent variables in Taguchi robust design in the first step of process optimization. Temperature of cultivation and pH of the culture medium were chosen separately as the independent variables in CCD (the second step of process optimization).

The growth of *E. coli* and the production level of NAP in the starting unoptimized medium had been examined first. The time profiles of the cultivation of *E. coli* in this medium are given in Figure 1. The organism grew with no lag phase and reached the stationary phase after approximately 22 h of cultivation. The cultivation was however, continued upto 26 h and the sampling was done at hours 24 and 26 for NAP estimation. The growth ($OD_{590\ nm}$) at these time was 8.43 and 9.38 respectively. The corresponding cellular dry weight was 4.09 and 4.56 g l^{-1} respectively. The NAP production levels were 265.5 and 309.5 mg l^{-1} at hours 24 and 26 of cultivation respectively. The hour 24 data was considered as the basis for the optimization studies using Taguchi robust design.

The experimental results of Taguchi robust design are presented in Table 3 and the system behaviour is explained by a simple first order polynomial equation.

$$\widehat{Y} = 385.83 + 15.78X_1 + 71.26X_2 - 3.51X_3 \quad (3)$$

The function (production level of NAP) was maximized at x_1 (Glycerol) = 1.0 (58.97 g l^{-1}), x_2 (Na-polyphosphate) = 1.0 (12.0 g l^{-1}) and x_3 ($MgSO_4 \cdot 7H_2O$) = −1.0 (2.0 g l^{-1}).

The time profiles of the cultivation of *E. coli* and the profiles of controlled physical parameters in this optimal medium are given in Figure 2. The growth ($OD_{590\ nm}$) at hours 24 and 26 were 17.5 and 18.8, respectively. The corresponding cellular dry weight was 8.51 and 9.14 g l^{-1} respectively. The NAP production levels were 469.4 and 649.2 mg l^{-1} at hour 24 and at 26 respectively. The optimization of the medium components resulted in almost a doubling of the cellular growth and the NAP production level was increased by 76.8% at hour 24 of cultivation.

The statistical analysis of these experimental results showed that Na-polyphosphate had a profound effect on NAP production. It is also evident from the value of the corresponding coefficients ($\beta_2 = 71.25$) in the regression equation. The statistical analysis was further verified by experimentation, which also proved that the composition of the medium where, sodium polyphosphate concentration was the highest, resulted the maximum production of NAP. The cultivation time was further continued up to 30 h. It was observed that

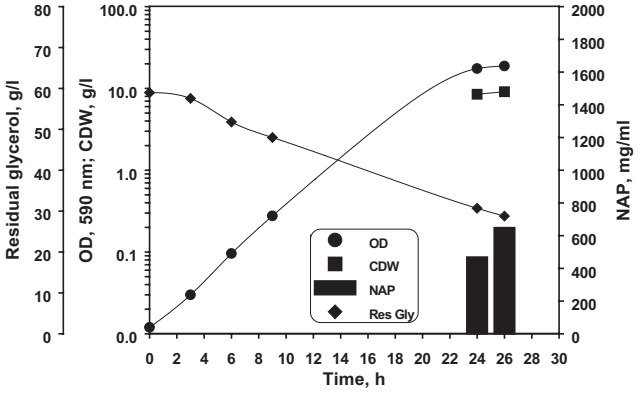

Figure 2. Time profiles of recombinant *E. coli*-NAP cultivation in optimized chemically defined medium obtained through medium component optimization using Taguchi robust design.

Table 3. Results of medium optimization using Taguchi robust design.

Experiment number	Glycerol[a], g l^{-1} (X_1)	$(NaPO_3)_7$, g l^{-1} (X_2)	$MgSO_4 \cdot 7H_2O$, g l^{-1} (X_3)	NAP, μg ml^{-1} (at 24th h)
1	19.66 (−1.0)	4.0 (−1.0)	2.0 (−1.0)	295.26
2	19.66 (−1.0)	12.0 (1.0)	4.0 (1.0)	444.8
3	58.97 (1.0)	4.0 (−1.0)	4.0 (1.0)	333.88
4	58.97 (1.0)	12.0 (1.0)	2.0 (−1.0)	469.37
Σ (−1.0)/2	370.03	314.54	382.32	
Σ (1.0)/2	401.63	457.09	389.34	Average 385.83
Optimal	(1.0)	(1.0)	(−1.0)	

The coded values (coordinates) are given in the parenthesis.

[a] The concentration of glycerol was varied in order to obtain C to N ratio of 5 to 15.

Table 4. Experimental design and responses (experimental and predicted) for temperature and pH optimization.

Experiment number	x_1	x_2	Y_{expt}	Y_{pred}
1	−1	−1	486.71	560.9
2	1	−1	980.69	985.9
3	−1	1	382.62	268.7
4	1	1	9.7	0
5	0	0	813.21	808.6
6	0	0	777.14	808.6
7	−1.414	0	121.01	127.0
8	1.414	0	11.56	115.0
9	0	−1.414	1292.24	1213.6
10	0	1.414	0.0	187.5
11	0	0	884.51	808.6
12	0	0	759.69	808.6

though the micro-organism reached stationary phase of its growth profile, the NAP production level continued to increase. The production level of recombinant NAP at hour 30 was 780.7 mg l^{-1}.

Since temperature of cultivation and the pH of the medium have substantial influence on microbial physiology and on recombinant protein production, an elaborate study was conducted in order to find optimal conditions for the enhanced growth of *E. coli* and to further maximize NAP production. Central composite design and RSM strategies were adopted as an efficient way to find optimal culture conditions. The temperature of cultivation and the pH of the production medium were chosen as the independent variables in the experimental design. The NAP production level at hour 30 of cultivation was considered as the dependent variable. A design with 12 experimental combinations was carried out in a sequential manner. The intracellular NAP production level was estimated in hour 30 samples originating from these experiments. The combinations and the corresponding NAP production levels are given in Table 4. The experimental results were subjected to multiple regression analysis and the NAP production was described in terms of a full second order polynomial model (Equation (4)).

$$\widehat{Y} = 913.559 - 4.216x_1 - 362.823x_2 - 343.994x_1^2 - 54.077x_2^2 - 216.725x_1x_2 \quad (4)$$

The predicted NAP production levels were then computed using this equation for each experimental combination in the design and were displayed together with the experimental values in Table 5. This model was further tested for adequacy by the analysis of variance and the summary of ANOVA is presented in Table 5. The effect of temperature and pH on NAP production level is depicted in a pseudo-three dimensional response surface plot (Figure 3).

Since the main objective of the RSM was to obtain an optimal combination which could give the maximum intracellular NAP production under the experimental conditions investigated, Equation (4) was mathematically optimized using Matlab (Mathworks Inc., USA). The optimal values of temperature and pH of cultivation were found to be 37.2 °C and 6.3, respectively.

Table 5. Analysis of variance (optimization of temperature and pH).

Sum of squares (SS)	Value
$(SS)_{Total}$	5,654,500
$(SS)_{Mean}$	3,541,500
$(SS)_{Corrected\ for\ the\ mean}$	2,113,000
$(SS)_{Factor}$	1,999,200
$(SS)_{Residual}$	113,220
$(SS)_{Pure\ Error}$	9165.5
$(SS)_{Lack\ of\ Fit}$	104,050
R^2	0.95
F	21.19
P	0.00095

To verify this result of optimization, a set of experiments was conducted using the optimal culture conditions and the result was compared with the theoretical value. The time profiles of *E. coli* cultivation using this optimal condition is given in Figure 4.

The statistical analysis of the data obtained from CCD showed that pH has a greater influence on both cellular growth and on NAP production level. Acidic pH and higher temperature favoured the enhancement of NAP production. However, the quadratic effect of temperature was found to be substantial. It is also important to note that the interaction effect of temperature and pH was conspicuous ($\beta_{12} = 216.725$). The numerical values of regression coefficients are further supported by the response surface plot of NAP production level as the function of temperature and pH (Figure 3). The combined effect of these two independent variables is described by a curved surface, suggesting that there is a stationary point which has the maximum estimated response. The regression equation is able to take into account 95% ($R^2 = 0.95$) of the total variation due to multiple independent variable. The relatively higher value of R^2 suggests the model is a good

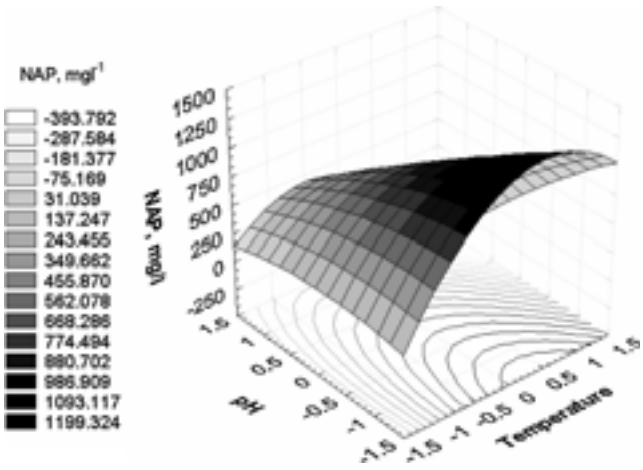

Figure 3. Pseudo-three-dimensional response surface plot: combined effect of temperature of cultivation and pH of cultivation on recombinant NAP production in *E. coli*.

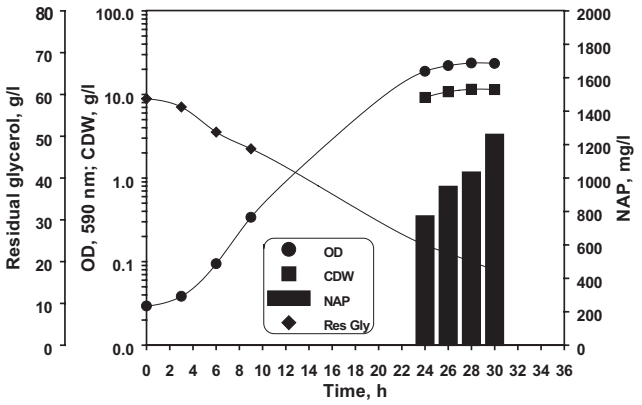

Figure 4. Time profiles of recombinant *E. coli*-NAP cultivation in optimized chemically defined medium using optimal temperature and pH of cultivation.

predictor of the target function (NAP production level). The multiple regression equation was maximized with in the boundary of axial points ($\alpha = \pm 1.414$). The optimal combination (coded) was found to be 0.4393, −1.414 for temperature and pH respectively. The actual values of temperature and pH were 37.2 °C and 6.3. The maximum theoretical NAP production level was 1280 mg l^{-1}. The optimization result was verified by conducting a set of experiments with both unoptimized and optimized culture conditions. The cultivation profile with optimized temperature and pH (Figure 4) revealed a better cellular growth ($OD_{590\ nm} = 26.0$ at hour 30) in comparison to that in un-optimized conditions ($OD_{590\ nm} = 21.0$ at hour 30). The NAP production level in optimized conditions was 1261 μg ml^{-1} at hour 30 which was 61.5% higher than that in unoptimized culture conditions (Figure 5). This is an achievement of optimization studies on cultivation conditions. Since

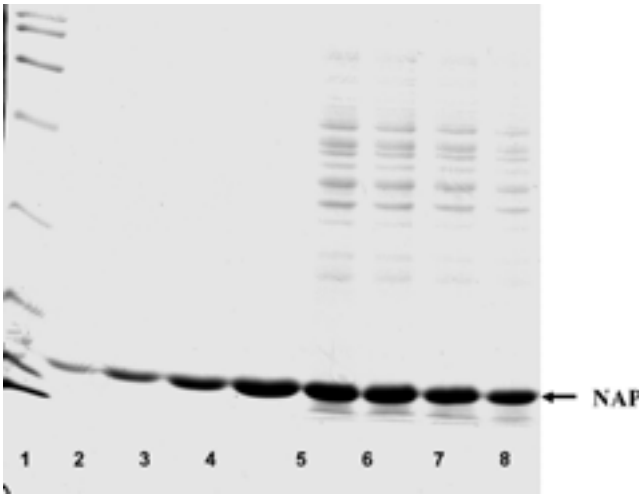

Figure 5. Detection and quantification of NAP in SDS-PAGE (lane 1: Molecular weight marker, lane 2–5: purified NAP in different concentrations, lane 6: optimized conditions-NAP production level at hour 26, lane 7: optimized condition-NAP production level at hour 24, lane 8: unoptimized condition-NAP production level at hour 26 and lane 9: unoptimized condition-NAP production level at hour 24.

experimental NAP production level in optimized culture conditions is very close to that of the theoretical maximum level (−1.02% difference), the regression equation can be designated as an accurate predictive model. An overall 2.91-fold increase in recombinant NAP production level was obtained at hour 24 of cultivation through process optimization.

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