Sequential simplex optimization of recombinant biotinylated survivin production by *Escherichia coli* using mineral supplementation

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ABSTRACT

The sequential simplex method is a useful tool for rapidly optimizing a process by moving through factor space via a relatively simple geometric algorithm. This method was employed to optimize growth and survivin-BCCP production in *Escherichia coli* Origami B using zinc sulphate supplemented culture medium. Five experimental parameters were investigated: concentration of zinc sulphate and IPTG, pH, temperature, and agitation rate. Optimized conditions were as follows: 190 µM zinc sulphate with 246 µM IPTG, pH 7, at a temperature of 23.5 °C and agitation rate of 345 rpm. The results for cell density and survivin-BCCP production were, 17% and 140% higher than in non-zinc supplemented medium and 12.6% and 25.5% higher than could be obtained by varying one-factor-at-a-time. The present work demonstrates the effectiveness of zinc sulphate supplementation and the efficiency of the sequential simplex optimization method in obtaining high yields.

1. Introduction

Survivin is an apoptosis inhibitor that has been shown to play a role in cell cycle regulation [1]. The encoding region of this cytoplasmic protein is localized on chromosome 17q25 [1,2]. Survivin is a structurally and functionally unique member of the inhibitor of apoptosis protein (IAP) gene family. It contains a single baculoviral IAP repeat (BIR) and an extended-COOH terminal alpha helix. In solution it forms a dimer and exhibits cell cycle regulated expression that peaks at mitosis. Survivin is expressed in cells during the G2/M phase of the cell cycle and its mRNA and protein are degraded at G1 by ubiquitin-dependent proteasome [3]. IAP molecules are characterized by the presence of one or more copies of a zinc binding fold with an approximate length of 70 amino acids, termed BIR. Survivin is regulated in transcriptional processes and can be detected in the majority of human cancers, but not in adult normal tissues [4].

Our laboratory has previously examined many aspects of survivin, such as the production of monoclonal antibodies against survivin for early cancer diagnosis. Recently, Tayapiwatana et al. [5] were able to produce *in vivo* survivin-BCCP–biotin (SVV-BCCP) from *Escherichia coli* Origami B strain for the purpose of monoclonal antibody production. The biotin–avidin/steptavidin system is used in numerous biotechnological and diagnostic applications, due to the high affinity of the proteins avidin and streptavidin to small biotin molecules [6]. According to a former report, Zn^{2+} ions are linked tetrahedrally by Cys 57, Cys 60, His 77 and Cys 84 bridges in the core beta-sheet with alpha4 and alpha5 helices in the survivin structure [3,4].

Recombinant microorganism culture conditions are typically optimized by the one-factor-at-a-time strategy, which involves varying the levels of one factor while keeping all others constant. This strategy, however, ignores the possible interactions between variables. In addition, it involves a relatively large number of experiments when several factors are included. Other strategies, such as a factorial design, utilize mappings in factor space, but an even larger number ($n!$) of experiments are required. In contrast, sequential simplex optimization [7] is useful for rapidly optimizing processes by moving through a factor space via a relatively simple geometric algorithm. Each movement involves changing all factor levels, but factor interactions are accommodated in this scheme. This method has been demonstrated to be an efficient strategy for rapid optimization of multifactor systems in chemistry [8,9] and is being increasingly applied in biochemistry [9–11]. The objective of this study was to apply the sequential simplex method in order to opti-

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mize recombinant biotinylated SVV-BCCP production by *E. coli* in mineral ion supplemented medium.

2. Materials and methods

2.1. Microorganisms and media

E. coli Origami B harboring the pAK400cb-SVV expression vector was obtained as described previously [5]. The microbe was cultured in modified super broth medium (SB medium) (30 g/L tryptone, 15 g/L yeast extract, and 10 g/L MOPS, pH 7.0) supplemented with glucose (0.05%, w/v), tetracycline (10 μg/mL), chloramphenicol (25 μg/mL), kanamycin (15 μg/mL), isopropyl-beta-D-thiogalactopyranoside (IPTG, 100 μM), and 4 μM D-biotin (Sigma, St. Louis, MO). The culture was incubated at 25 °C, 180 rpm for 22 h. Bacterial cells were harvested by centrifugation at 4000 × g, 4 °C for 10 min and resuspended in B-PER II extracting reagent (Pierce, Rockford, USA). The bacterial extract containing biotinylated SVV-BCCP was kept at −70 °C.

2.2. Fermentation substrates and mineral supplements

Experiments were conducted to determine the type of supplemented mineral cation in the modified SB medium that would result in the optimal production of biotinylated SVV-BCCP by Origami B. The minerals supplemented in culture media were $ZnCl_2$, $ZnSO_4$, $CuSO_4$, $CaCl_2$, $MnCl_2$, $FeSO_4$ and $MgCl_2$ at 200 μM. Bacterial cells were harvested and final OD at 600 nm was measured.

2.3. Optimization experiments

2.3.1. Culture conditions by varying the one-factor-at-a-time (OF method)

Experiments were conducted to determine the optimal conditions for biotinylated SVV-BCCP production by *E. coli* Origami B. The following $ZnSO_4$ concentrations were used: 50, 100, 150, 200, 250, 300, 350 and 400 μM. The other parameters that were varied included: pH of 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5; incubating temperatures of 20, 21, 22, 23, 24, 25, 26, 27 and 28 °C; optical density (OD) of cells for induction of 0.5, 0.7, 1.0, 1.2, 1.7, 2.0, 2.2 and 2.5; IPTG concentrations of 100, 150, 200, 250, 300, 350, 400, 450 and 500 μM; agitation rates of 200, 220, 240, 260, 280, 300, 320, 340 and 360 rpm; cultivation times of 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 h. Each experiment was carried out in triplicate.

2.3.2. Culture conditions for sequential simplex optimization (SS method)

A 5 L fermentor (Minifor, Infors-ht, Switzerland) with a working volume of 1.5 L was used for this study. Medium composition and fermentation parameters were varied according to the experimental design. The liquid cultures were constituted using $ZnSO_4$ (0–400 μM) and IPTG (100–500 μM). The ranges of the other fermentation parameters for simplex optimization were as follows: incubating temperature, 15–30 °C; agitation rate, 100–600 rpm; optical density of cells for induction (OD 0.5–2.5). Each experiment was carried out in triplicate.

2.4. Detection of biotinylated SVV-BCCP protein by ELISA

A micro titer plate was coated with 0.5 μg of egg white avidin (Sigma) in 50 μL of carbonate/bicarbonate buffer (pH 9.6) at 4 °C for 18 h. The coated wells were blocked with 2% skim milk in PBS for 1 h at room temperature. After being washed three times with washing buffer (0.05% v/v Tween 20 in PBS pH 7.2), 5 μg of bacterial extract from pAK400cb-SVV transformed Origami B in 50 μL of 2% skim milk in PBS was added into each avidin-coated well. After

incubation at room temperature for 1 h, the plate was washed three times with washing buffer. The bound SVV-BCCP was traced by adding 50 μL of 10 μg/mL mouse monoclonal anti-survivin (clone 3) as described previously [5]. Subsequently, HRP conjugated rabbit anti-mouse immunoglobulin antibody (DAKO, Hamburg, Germany) was added. After 1 h incubation and washing, TMB color substrate (Invitrogen, Wisconsin, USA) was applied to each well. The plate was incubated at room temperature for 15 min for color development. The reaction was stopped by adding 1 N HCl and the optical density was measured at 450 nm.

2.5. SDS-PAGE and Western immunoblotting

Bacterial extract containing biotinylated SVV-BCCP was separated by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel. The separated proteins were electroblotted onto a PVDF membrane. The membrane was blocked at 4 °C for 18 h in 5% skim milk in PBS (pH 7.2), and then incubated with mouse anti-survivin mAbs and followed by HRP conjugated rabbit anti-mouse immunoglobulin antibodies (DAKO, Hamburg, Germany) for 1 h at room temperature on a shaking platform. After washing four times with 0.05% v/v Tween 20 in PBS (pH 7.2), the immunoreactive bands were visualized by a chemiluminescent detection system (Pierce, Rockford, USA).

2.6. Experimental design

The five parameters to be varied were: concentration of $ZnSO_4$, concentration of IPTG, optical density of cells for induction, temperature of incubation, and agitation rate.

For the initial one-variable-at-a-time experiment, the values of the four of the five factors were held constant near the middle of their ranges. The first factor was then varied until an optimum was reached. This optimum for the first variable was then used while the second factor was varied, etc.

For variable-sized simplex optimization, the initial simplex was selected by choosing six ($n + 1$) experimental designs that covered a wide range of values in the factor space. The responses of the experiments were ranked according to the definitions: best response (B), next to the worst response (NW), concentration on the worst side (Cw) and reflection (R). The vertex (the condition calculated by sequential simplex optimization method) that produced the worst response in the initial simplex was replaced by a new set of factors for the next experiment in *n*-space by reflecting through the centroid of the remaining plane in hyperspace (formed by the *n* remaining vertices). Reflections, expansions and contractions were accomplished by following the variable-sized simplex rules [10,11]. Boundary violations were handled by assigning the worst response to the results of that experiment and proceeding to calculate the next set of factor levels to be tried as a Cw contraction.

3. Results and discussion

In the development of an optimized process for the enhanced production of recombinant biotinylated SVV-BCCP, selecting a suitable mineral supplemented medium and establishing the most favorable fermentation conditions are the two most important components. The culture medium was supplemented with cation minerals at a concentration of 200 μM (see Fig. 1.).

The response function shows that $ZnSO_4$ was the best mineral for enhancing biotinylated SVV-BCCP production. The relative amount of recombinant protein produced from $ZnSO_4$ supplemented medium was 2.26 OD whereas 1.12 OD was detected in control medium by avidin captured ELISA method at λ 450 nm. Sulphur ions are essential in anabolism of sulphur containing amino acids, e.g. cysteine [11]. Since the SVV molecule contains six CYS