Effect of aeration and agitation on the protease production by *Staphylococcus aureus* mutant RC128 in a stirred tank bioreactor

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Received: 29 November 2007 / Accepted: 6 May 2008 / Published online: 20 May 2008 © Springer-Verlag 2008

Abstract  The modified rotating simplex method has been successfully used to determine the best combination of agitation rate and aeration rate for maximum production of extracellular proteases by *Staphylococcus aureus* mutant RC128, in a stirred tank bioreactor operated in a discontinuous way. This mutant has shown altered exoprotein production, specially enhanced protease production. Maximum production of proteases (15.28 UP/ml), measured using azocasein as a substrate, was obtained at exponential growth phase when the bioreactor was operated at 300 rpm and at 2 vvm with a volumetric oxygen transfer coefficient ($K_{La}$) of 175.75 h$^{-1}$. These conditions were found to be more suitable for protease production.

Keywords  Aeration · Agitation · Proteases · Rotating simplex method · *Staphylococcus aureus*

Introduction

Different optimization techniques are used to determine the suitable operating parameters [1, 2]. The most commonly used method in bioprocess is the one-factor-at-a-time but this strategy is laborious and time consuming, especially when several factors are included. Besides, it ignores possible factor interactions [3, 4]. The statistical methods reduce the number of experiments needed and consider the interaction among variables [2, 5]. However, these methods also have disadvantages, in that many experiments cannot be conducted simultaneously in bioreactors.

The simplex sequential method and its modifications is one of the most popular non-statistical optimization techniques. This method has been successfully used for chemical systems, but in the past years it has been applied to biosystems, specially those with bioreactors where the experiment cannot be performed in groups [4, 6] The most important advantages of the simplex method are simplicity, efficiency and sequential character, which means that a new trial is determined by the result of the latest trial [1].

Several microbial fermentations are affected by cultural conditions. Particularly, it is well known that extracellular protease production by microorganisms in bioreactors is greatly affected by media components, physical factors such as, aeration, agitation, dissolved oxygen, temperature, inoculum density and incubation time [7].

Oxygen shows diverse effects on product formation in aerobic fermentation process by influencing metabolic pathway and changing metabolic fluxes [8]. It has been observed that the respiration rate of aerobic microbe is generally independent of dissolved oxygen above a certain critical level. However, below that level, a small change in the dissolved oxygen may cause a significant physiological alteration in cell metabolism [9]. Supply of oxygen to the growing cell population is the rate-limiting step in many aerobic fermentation processes due to poor solubility of oxygen in the culture medium, which is determined by the oxygen transfer rate and is governed by the volumetric oxygen transfer coefficient ($K_{La}$), one of the most important parameters in scaling-up aerobic fermentation processes.
Several reports have demonstrated that the presence of oxygen is essential for bacteriolitic enzymes, nuclease, enterotoxins and lipase production by *Staphylococcus aureus* [10–12] but little is known about the effect of oxygen in the production of proteases from this microorganism. *S. aureus* produce different types of proteases widely studied and classified into three types; serine, thiol and metallo proteases [13]. Nowadays the economic importance of metallo proteases in some application, especially as an anti-inflammatory agent is well known [14].

In a previous work [15], reported the isolation and characterization of a Tn925 induced mutant of *S. aureus*, designated RC128, altered in exoprotein production, specially in enhanced production of proteases. Partial protease purification and further characterization showed the presence of two major fractions of proteolytic activity corresponding to serine and metallo proteases as measured by PMSF and 1,10-phenanthroline and EDTA inhibition, respectively (data not published).

The aim of the present work was to identify the best combination of agitation rate and aeration rate by means of the application of the modified simplex method, for maximization of protease production in the mutant strain *S. aureus* RC128 in a lab scale bioreactor. The effect of *K*La on the process was also studied.

**Materials and methods**

**Microorganism and media**

The strain used for protease production was *S. aureus* RC128, a protease hyperproducer, tetracycline-resistant mutant derived from *S. aureus* RC108 obtained by insertion of Tn925 [15]. The strain was maintained on Brain Heart Infusion (BHI, Difco), cultured for 18 h at 37 °C and stored at −20 °C with 0.8% glycerol until its use.

**Culture conditions**

For each assay an aliquot of the frozen culture of the mutant was grown on 15 ml BHI with tetracycline at 5 μl/ml during 24 h, at 37 °C. This culture was grown in Erlenmeyer flasks with 250 ml BHI in a rotary shaker 250 rpm with 5 cm amplitude at 37 °C for 14 h.

**Experimental conditions**

Protease production was carried out in the 6.5 l stirred tank bioreactor Bioflo 2000 (New Brunswick Scientific Co., Inc., USA) with a working volume of 5 l. The bioreactor was equipped with two 6-bladed Rushton turbine, controllers for stirring speed, pH, temperature and dissolved oxygen concentration. The bioreactor was operated in a discontinuous way with respect to the liquid phase, and continuously with regard to the gas phase (air). The pH was controlled with Brodley James sensor by adding 4 M NaOH or 4 M HCl to the required value (at pH 7–7.1). The dissolved oxygen concentration was measured using a Mettler Toledo sterilizable/autoclavable O2 sensor; the temperature was maintained at 37 °C; Antifoam A (Sigma) used as antifoam agent.

Protease activity was monitored at the agitation rate between 200 and 600 rpm, and the aeration rate between 1 and 2.75 vvm. Each experiment was run for a period of 30 h, and samples were collected, for biomass, enzymatic activity and total protein determination. All experiments were done at least twice.

**Proteolytic activity**

Proteolytic activity was measured using azocasein (Sigma Chemical Co., St Louis, MO, USA) as the substrate following [16] methods with slight modifications. Briefly, a volume of 1,600 μl of azocasein (3 g/l) in 50 mM Tris-HCl buffer pH 7.4 was added to a 600 μl of the supernatants culture and incubated at 45 °C for 1 h. The reaction was stopped by adding of 800 μl of 10% trichloroacetic acid (TCA). After centrifugation, the optical density of supernatant was determined at 440 nm. One unit of proteolytic activity (UP) was defined as an increase of 0.1 optical density after 1 h incubation at 45 °C.

**Cell growth**

Cell growth was determined by optical density measures at 600 nm. They were correlated with dry weight (g/l) by a calibration curve. The growing parameters (maximum specific growth rate and maximum cellular concentration) were determined by a logistic model [17]:

\[
\frac{dX}{dt} = \mu_{\text{max}} X \left(1 - \frac{X}{X_{\text{max}}} \right)
\]

where

- \(X\) cell concentration (g/l)
- \(X_{\text{max}}\) maximum cell concentration (g/l)
- \(\mu_{\text{max}}\) maximum specific growth rate (h⁻¹)
- \(t\) time (h)

**Determination of protein**

The protein contents of samples were determined according to [18] protocol. For this, 1 ml of Bradford reactive was added to 100 μl of supernatants and incubated for 5 min at
room temperature, and optical density read at 595 nm. Bovine serum albumin was used as standard.

Optimization procedure

For optimization of the variables, agitation rate and aeration rate for proteases production, the simplex method modified by Nelder and Mead [19] was used.

This self-directing optimization is one of the widely used non-statistical techniques where experiments cannot be performed in groups and can be used either to maximize or minimize the response. The objective of the sequential simplex method is to drive a geometric figure, simplex, with \( n + 1 \) vertices, where \( n \) is the number of analyzed factors, toward the region of the factor space that is of optimum response.

The procedure starts with a set of experiments. The levels of the variables for these combinations are fixed taking into account previous results (data not shown). The worst combination is rejected, and blending the best of the others generates a new experiment. This iteration is repeated until no further improvement in yield is observed.

Nelder and Mead [19] modification allowed this simplex to expand in directions that are favorable and to contract in directions that are unfavorable. In this case the designs begin with a group of three experiments and can be visualized as a triangle and continued the movement in the response plane (proteases production). The movements of the modified simplex method are expressed in the following equation and shown in a two-dimensional surface plot representing the joint variation of agitation rate and aeration rate for each experimental run (Fig. 1):

\[
P = \left( \frac{B_1 + B_2}{2} \right) + b \left[ \left( \frac{B_1 + B_2}{2} \right) - W \right]
\]

where:

- \( P \) new experimental combination
- \( B_1, B_2 \) the two best points from the last three experimental runs
- \( W \) the worst point from the last three experimental runs
- \( b \) coefficient of movement of the simplex, \( (b = 1 \) reflection coefficient, \( b = 2 \) expansion coefficient, \( b = 1/2 \) contraction coefficient and \( b = -1/2 \) contraction coefficient with change of direction)

Evaluation of the mass transfer coefficient \( K_{La} \)

The volumetric oxygen transfer coefficient referred to the liquid phase volume was determined experimentally under production conditions for \( S. aureus \) RC128 strain using the dynamic method developed by Bandyopadhyay et al. [20]. In brief, the technique consists in following the evolution of the oxygen dissolved in the culture medium during a short interruption and further resumption of the airing supplied to the bioreactor.

The \( K_{La} \) values were determined from the data of transient dissolved oxygen obtained during degassing and aeration periods. The probe response was corrected by the first-order lag model [21]. The equation used for \( K_{La} \) calculation is shown below:

\[
C_E^E = A + \frac{B}{1 - K_{La}\tau_E} e^{-K_{La}\tau_E} + C e^{-\tau_E}
\]

where

- \( C_E^E \) oxygen concentration shown by probe (g/l),
- \( B, C \) integration constants
- \( \tau_E \) probe time constant (h)
- \( A \) parameter: \( C_{O_2}^s - \frac{Q_{O_2} X}{K_{La}} \)
- \( C_{O_2}^s \) saturation concentration of dissolved oxygen (g/l)
- \( Q_{O_2} \) specific oxygen uptake rate (g/g.h)

In order to determine the \( \tau_E \) value the technique of [22] was used.

To evaluate the experimental data, Eq. (3) is used to approximate the registered oxygen composition values, considering the variables \( A, B, C \) and \( K_{La} \) as adjusting parameters to minimize the objective function given in Eq. (4):

\[
\Psi(A, B, C, K_{La}) = \sum_{i=1}^{m} \left( C_{E_{Exp}}^E - C_{E_{Pred}}^E \right)^2
\]

The parameters \( A, B, C \) and \( K_{La} \) in Eq. (3) were determined by means of a multiparametric non-linear regression technique.
Results and discussion

Effects of aeration and agitation rates on growth and proteases production

In this study, the modified simplex optimization technique was used to determine the best combination of aeration rate and agitation rate to maximize the proteolytic enzyme production by S. aureus RC128 in the batch stirred tank bioreactor. The pH and the temperature of production medium were set up at 7 and 37 °C, respectively; according to previous experiments carried out in shake flask cultures (data not shown). The initial range of the variables was as follows, aeration rate: 1 and 2 vvm and agitation rate: 200, 300 and 500 rpm.

A large initial simplex was select to cover a fairly broad range. The response from one of the vertexes could even be close to the optimum as was observed in this work.

A total of six experiments were necessary to obtain the best combination of agitation rate and aeration rate. The results of optimization procedure are shown in Table 1. After the first three experiments, protease activity of run 1 was the lowest. The next experiment (run 4) was performed at conditions obtained after following the rule of modified simplex. The following simplex was performed by the experiments not rejected (run 2 and 3) and by run 4. After several simplex, it was not possible to improve the yield observed in run 3 (15.28 UP/ml). After several simplex, the yield obtained in the run 3 (15.28 UP/ml) was not possible to be improved since the application of modified simplex rule conducted to the operative conditions employed in this run. Thus, the better conditions by simplex modified method were aeration rate: 2 vvm and agitation rate: 300 rpm. A combination of high aeration rate and high agitation rate (run 4) did not give a maximum protease activity. Similarly, a combination of low agitation rate and low aeration rate (run 1) did not favor protease activity either. These observations indicate that a proper balance between aeration rate and agitation rate was required for an efficient proteases production. These operative conditions also gave the maximum biomass concentration (9.86 g/l) even though the maximum specific growth rate (0.752 ± 0.021 h⁻¹) was observed at 2 vvm and 600 rpm (Table 1). It is well known that slightly high aeration rate improves enzyme synthesis because it is able to maintain a significative dissolved oxygen level in the culture medium. For an optimal enzyme production, it is also necessary to reach a good mix of the culture broth since agitation produces a dispersion of air in the culture medium, homogenizes the temperature and the pH and improves transference rate of nutrients. However, high speeds of agitation are against the enzymatic activity, probably due to the shear stress caused by the blade tips of the impeller, which increase as the revolution speed increases [23]. Stress condition may contribute negatively toward cell growth and enzyme stability [24].

Effects of $K_{La}$ on growth and proteases production

$K_{La}$ is an important parameter since it describes the aeration capacity of the fermentation system and supplies information for the scale-up process. $K_{La}$ was determined experimentally at each combination of agitation rate and aeration rate using the dynamic method. $K_{La}$ values increase as agitation rate and aeration rate increase (Table 1). Our results are in agreement with previous observations in mechanically agitated vessels, where aeration affects $K_{La}$ more strongly than aeration [25]. The effect of $K_{La}$ on the maximum proteolytic activity and the maximum specific growth rate is shown in Fig. 2. As it is observed, a general tendency of maximum specific growth rate essentially increased with increasing $K_{La}$ values, demonstrating that growth was dependent on oxygen supply. There was no such correlation between the maximum proteases activity and $K_{La}$. The level of protease activity reached a maximum value of 15.28 UP/ml at $K_{La}$ 175.75 h⁻¹ (300 rpm–2 vvm) and then decreased with further increase in $K_{La}$. It is clearly shown that an increase in $K_{La}$ alone does not facilitate the maximum protease activity.

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Aeration rate (vvm)</th>
<th>Agitation rate (rpm)</th>
<th>Maximum protease activity (UP/ml)</th>
<th>Maximum biomass (g/l)</th>
<th>Maximum specific growth rate (h⁻¹)</th>
<th>$K_{La}$ (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>200</td>
<td>4.90</td>
<td>6.59</td>
<td>0.388 ± 0.031</td>
<td>58.36</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>500</td>
<td>12.49</td>
<td>8.79</td>
<td>0.603 ± 0.011</td>
<td>211.82</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>300</td>
<td>15.28</td>
<td>9.86</td>
<td>0.574 ± 0.035</td>
<td>175.75</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>600</td>
<td>10.48</td>
<td>7.35</td>
<td>0.753 ± 0.022</td>
<td>305.32</td>
</tr>
<tr>
<td>5</td>
<td>1.75</td>
<td>500</td>
<td>10.87</td>
<td>7.61</td>
<td>0.702 ± 0.023</td>
<td>228.73</td>
</tr>
<tr>
<td>6</td>
<td>2.75</td>
<td>300</td>
<td>13.01</td>
<td>8.29</td>
<td>0.660 ± 0.018</td>
<td>189.20</td>
</tr>
</tbody>
</table>
Under all operative conditions tested, the protease activity as a time function is shown in Fig. 3. It is clear from this figure that between 4 and 15 h enzyme production is higher when the bioreactor was operated at 300 rpm and at 2 vvm with a $K_{La} = 175.75 \text{ h}^{-1}$. For each condition of fermentation the highest activity was obtained at 6 h during the exponential growth phase (Fig. 4). Similar results were observed by Arvidson et al. [10] for *S. aureus* bacteriolytic enzymes production and by Carpenter and Silverman [11] for staphylococcal nuclease enzyme production. In all operative conditions, a progressive decrease in protease activity was observed after maximum activity had been achieved (Fig. 3). Biomass concentration profiles, proteolytic activity and total protein concentration for the best conditions (2 vvm, 300 rpm, $K_{La} = 175.75 \text{ h}^{-1}$) are shown in Fig. 5. The progressive decrease in protease activity could be explained by the hydrolysis of the enzyme by the protease itself because of protein concentration decrease (Fig. 5). Many researches have reported similar protease degradation beyond maximum enzyme production [26–28].

The present work demonstrated that the rotating simplex optimization technique was an efficient tool to determine the best combination of agitation rate and aeration rate for maximum production of extracellular proteases. It was found that aeration and agitation levels have a significant effect to improve the protease production by *S. aureus* RC128.

**Fig. 2** Effect of $K_{La}$ on the maximum proteolytic activity and the maximum specific growth rate

**Fig. 3** Protease production under different oxygen transfer coefficients

**Fig. 4** Cell concentration under different oxygen transfer coefficients

**Fig. 5** Time profiles of cell concentration, proteolytic activity and total protein of *S. aureus* in optimal condition (300 rpm–2 vvm $K_{La} = 175.75 \text{ h}^{-1}$)
Acknowledgments. The authors wish to thank the support given by the Secretaría de Ciencia y Técnica Universidad Nacional de Río Cuarto (UNRC), Argentina.

References