

**Optimization of the culture medium composition using response
surface methodology for new recombinant cyprosin B production in
bioreactor for cheese production**

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Abstract

The optimization of culture medium composition was carried out for improvement the recombinant cyprosin B production, an enzyme with high milk-clotting activity. Response surface methodology (RSM) was applied to evaluate the effect of variables namely glucose, yeast extract (YE) and bactopectone present in the culture medium, used for recombinant cyprosin B production by transformed *Saccharomyces cerevisiae* BJ1991 strain in shake-flask and bioreactor culture conditions. The central composite experimental design (CCD) was adopted to derive a statistical model for optimizing the composition of the fermentation medium. The optimal concentration estimated for each variable related to a theoretical maximum of cyprosin B activity (488 U mL^{-1}) was 30 g L^{-1} glucose, 15 g L^{-1} YE and 27 g L^{-1} bactopectone. The optimized medium composition, based on empirical model, led to a cyprosin B activity of 519 U mL^{-1} , which corresponds to an increase of 46%. The fermentation using optimized culture medium in a 5-L bioreactor allowed a significant increase in biomass (82%) and recombinant cyprosin B production (139%). The improvement in the recombinant cyprosin B production after optimization process can be considered adequate for large-scale applications, and the clotting activity of cyprosin B account for their use in industrial cheese making.

Keywords: Culture media composition, Cyprosin B, Fermentation, Response surface methodology, *Saccharomyces cerevisiae*

Introduction

Cyprosin B is an aspartic protease present in the pistils of *Cynara cardunculus*, with great potential for cheese production due to its high clotting activity [1]. The *S. cerevisiae* BJ1991 strain was transformed by CYPRO11 gene [2], which codifies for cyprosin B (International patent WO 00/75283, PCT/PT00/00007). The expression of CYPRO11 gene is controlled by GAL7 promoter which is inducible by galactose present in the culture medium. Studies on the expression and characterization of recombinant cyprosin B in bioreactor were previously carried out using transformed *S. cerevisiae* W303-1A, proving that active cyprosin B is efficiently expressed and secreted to the extracellular medium, having the ability for clotting milk [3].

The biochemical and nutritional cell environment in a bioreactor is strongly influenced by growth conditions and medium composition, namely carbon and nitrogen sources, mineral salts, trace elements, peptides, amino acids, vitamins and/or other factors. Therefore, the optimization of culture medium composition is essential for the effective production in bioreactor [4, 5]. The production of biomolecules, such as enzymes for food industry, often requires the use of complex and cheap industrial media based on yeast extract (YE) and bactopectone as nitrogen sources. Complex organic nitrogen sources are less expensive than pure amino acids and nitrogen bases and, additionally, can supply a variety of vitamins, minerals and other growth co-factors. Furthermore, cell growth is generally more rapid and efficient when these nutrients are used, since they reduce the number of components that cells would otherwise have to synthesize de novo. A widely used complex rich growth medium containing YE and bactopectone as nitrogen sources and glucose as carbon source played an important role in the efficiency and economics of the fermentation process [6]. The activity and stability of enzymes is influenced by the type of strain, cultivation conditions (temperature, pH, aeration, agitation, incubation time) and culture medium composition (particularly carbon and nitrogen sources) [7–9].

RSM is a mathematical and statistical technique for building empirical models that gained importance for optimizing production conditions of industrially high value products such as chemicals and enzymes and for studying enzyme kinetics. In this methodology, multivariate experiments are designed to reduce the number of assays necessary to optimize the process and to gather results more precise than those obtainable by univariate strategies. This statistical tool has been used in many biotechnological processes namely optimization of culture conditions [10–13]; enzyme

production [14, 15]; ethanol production [16, 17] and biomass production [18]. This statistical tool was also useful for optimization of recombinant products yields such as actinorhodin [19]; nisin [13]; lysozyme [20]; alkaline protease [12]; hirudin [21] as well as extracellular polysaccharose and biomass [22].

The aim of this work was to improve the production of recombinant cyprosin B by transformed *S. cerevisiae* BJ1991 strain applying the RSM tool for the optimization of culture medium composition's (glucose, YE and bactopectone) concentrations maintaining the inducer concentration (galactose) constant. These optimal experimental results were confirmed by shake-flask and bioreactor culture, and, furthermore, biochemical and milk-clotting studies were performed for process characterization.

Materials and methods

Microorganism

S. cerevisiae BJ1991 strain (MATa leu2, trp1, ura3-52, prb1-1122, pep4-3) was transformed previously with episomal plasmid (pCAF15), containing CYP11 gene (International patent WO 00/75283, PCT/PT00/00007). The stock of transformed cells was maintained in glycerol 50% (v/v) at -80 °C.

Inoculum and culture conditions

YNB medium [0.67% (w/v) yeast nitrogen base without amino acids (Sigma, Germany) and 2% (w/v) glucose (Merck, Germany)], supplemented with auxotrophics markers (70 mg L⁻¹ tryptophan and 20 mg L⁻¹ uracil), previously sterilized by microfiltration, was used as inoculum for *S. cerevisiae* BJ1991 culture [23]. The inoculum, containing 50 mL of YNB medium, was grown for 16 h in 200 mL shake-flasks maintained in an orbital shaker (200 rpm) at 30 °C (Aralab, Portugal) till reached the optic density (OD_{600nm}) of the 5.0. The experiments were carried out using shake-flasks (200 mL) containing 30 mL of culture medium, during 96 h (200 rpm) at 30 °C and 1.5 mL of inoculum in YNB medium. The culture medium is composed of glucose (Merck, Germany), bactopectone (Difco, USA) and YE (Merck, Germany) at different concentrations according to the experimental design (Table 2). The concentration of galactose (Sigma, Germany) was maintained constant (20 g L⁻¹) in all assays.

All culture media were buffered using a 50 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 chosen according to previous studies carried out in shake-flask (data not shown).

Enzyme production in bioreactor

The inocula for fermentations (control and optimized conditions) were grown overnight in a shake-flasks (500 mL) with 150 mL of YNB medium, at 30 °C (200 rpm) till OD_{600nm} (5.0). The fermentations were performed in a 5-L bioreactor (HT-Infors, Bottmingen, Switzerland) containing 3 L of culture medium at pH 6.0 and 30.0 °C. The original culture medium consists of 10 g L⁻¹ YE, 20 g L⁻¹ bactopectone, 20 g L⁻¹ glucose and 20 g L⁻¹ galactose; and for optimal concentrations, 15 g L⁻¹ YE, 27 g L⁻¹ bactopectone, 30 g L⁻¹ glucose and 20 g L⁻¹ galactose. The culture medium containing the complex nitrogen sources (YE and bactopectone) was sterilized in the reaction vessel at 121 °C for 20 min. The carbon sources (glucose and galactose) were added to complex nitrogen sources after sterilization, using a peristaltic pumping system in sterilized conditions. The bioreactor was inoculated at a 5% (v/v) ratio. The oxygen tension was measured as the percentage of dissolved oxygen in the culture medium in relation to air saturation, using an oxygen probe (Ingold, Germany). Along fermentation, the air was supplied at a constant flow rate of 3 L min⁻¹ (equivalent to 1 VVM). Mechanical agitation, produced by two Rushton turbines, was automatically controlled by setting the percentage of dissolved oxygen in the culture medium at 50%. The initial pH 6.0 was adjusted automatically during the fermentation by addition of acid (2 M HCl) and alkali (2 M NaOH) solutions. The samples were collected under aseptic conditions to analyze the fermentation performance, biomass, protein concentration and enzymatic activity evolution.

Analytical methods

Biomass determination

Biomass concentration was determined using a spectrophotometer (Shimadzu UV-1603, Japan) at an optical density of OD_{600 nm}. Cells were diluted in 0.8% (w/v) NaCl solution, and absorbance values were converted to dry cell weight (DCW) using a standard curve based on the weight of cell samples dried at 80 °C until constant weight.

Protein concentration

Protein concentration was determined according to the Bradford method using Coomassie reagent (Bio-Rad Laboratories, Hercules, CA, USA) (Bradford 1976) [24]. Bovine serum albumin (Sigma, Germany) was used as standard. The absorbance was measured in the wavelength at 595 nm in a spectrophotometer (Shimadzu UV-1603, Japan).

Enzymatic activity assay

Enzymatic activity of samples was evaluated using fluorescein isothiocyanate-labeled K-casein (FITC-casein) as the substrate, prepared according to the Twining method [25]. One unit of proteolytic activity was defined as the amount of enzyme that increases one unit of emitted fluorescence at 525 nm (excitation at 495 nm) after 1 h of hydrolysis at 37 °C [26]. Fluorescence intensity was measured using a spectrofluorimeter (Shimadzu RF-1501, Japan).

Efficiency of protein secretion

The biomass pellet collected after centrifugation at 8,500g for 5 min was resuspended and washed twice with 50 mM Tris–HCl containing 10 mM EDTA pH 8.3 buffer (TE buffer). Cell disruption was performed by vigorous mixing in TE buffer with 50% (v/v) 500- μ m glass spheres (Sigma, Germany) for 5 min with 1 min intervals in ice. Cell debris were removed by centrifugation at 8,500g during 15 min at 4 °C.

Milk-clotting activity

The clotting assay was carried out by adding 50 μ L of recombinant cyprosin B, obtained after each fermentation, to 1 mL of skim milk with 10 mM of CaCl_2 , pH 6.5 according to the International Dairy Federation method [27].

Statistical methods

Experimental design and optimization

RSM was used to study combined interactions among different physiological variables. The central composite design (CCD) was used to improve recombinant cyprosin B production, considering optimized levels of variables [28]. The substrates glucose (X_1 , g L⁻¹), YE (X_2 , g L⁻¹) and bactopectone (X_3 , g L⁻¹) were chosen as independent variables (Table 1). For a 2³ CCD with six axial points ($\alpha=(3)^{1/2}$) and two replicates at the center points of three factors, a set of 16 experiments was carried out. The control medium or original medium, defined by 10 g L⁻¹ YE, 20 g L⁻¹ bactopectone, 20 g L⁻¹ glucose and 20 g L⁻¹ galactose (YPGal-Glu medium), was used initially as base for this study. All variables were taken for three different coded levels: -1, 0 and 1. The minimum and maximum range, the variables studied and the full experimental plan, with respect to the coded and actual values, are listed in Tables 1 and 2. The statistical determination of the variable levels X_i was coded as x_i according to Eq. 1:

$$X_i = (x_i - x_{oi})/\Delta x_i \quad i = 0, 1, 2, \dots K \quad (1)$$

where X_i is (dimensionless) coded value of the real variable x_i , x_0 is the real value of X_i at the center point (zero) level and the Δx_i is the step change value.

Table 1 Experimental range and levels of independent variables

Variables	Range of levels (coded/actual)		
	−1	0	1
(X_1) Glucose (g L^{-1})	0.0	15.0	30.0
(X_2) Yeast extract (g L^{-1})	5.0	10.0	15.0
(X_3) Bactopeptone (g L^{-1})	5.0	17.5	30.0

Table 2 Central composite design matrix of independent variables with corresponding experimental and predicted value for cyprosin B activity

Run	Glucose (X_1)		YE (X_2)		Bactopeptone (X_3)		Cyprosin B activity (U mL^{-1})	
	Actual (g L^{-1})	Code	Actual (g L^{-1})	Code	Actual (g L^{-1})	Code	Experimental	Predicted
1	0.0	−1	5.0	−1	5.0	−1	180	319
2	0.0	−1	5.0	−1	30.0	1	176	356
3	0.0	−1	15.0	1	5.0	−1	357	474
4	0.0	−1	15.0	1	30.0	1	286	422
5	30.0	1	5.0	−1	5.0	−1	255	258
6	30.0	1	5.0	−1	30.0	1	412	434
7	30.0	1	15.0	1	5.0	−1	440	399
8	30.0	1	15.0	1	30.0	1	487	487
9	0.0	−1.68	10.0	0	17.5	0	261	397
10	40.2	1.68	10.0	0	17.5	0	425	400
11	15.0	0	1.6	−1.68	17.5	0	359	376
12	15.0	0	18.4	1.68	17.5	0	456	551
13	15.0	0	10.0	0	0.0	−1.68	178	270
14	15.0	0	10.0	0	38.5	1.68	356	376
15	15.0	0	10.0	0	17.5	0	352	418
16	15.0	0	10.0	0	17.5	0	353	418

After experiments, the average maximum recombinant cyprosin B activity was taken as the dependent variable or response (Y). A second-order polynomial equation was then fitted to the data by the multiple regression procedure. From this, an empirical model was obtained relating the response evaluated for the independent variables of the experiment. For a four-factor system, the model equation is:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (2)$$

where Y is the predicted response; β_0 is the intercept coefficient; β_1 , β_2 and β_3 are the linear coefficients; β_{11} , β_{22} and β_{33} are the quadratic coefficients; β_{12} , β_{13} and β_{23} are the interaction coefficients (Eq. 2).

Response surface plot

The quadratic model obtained from regression analysis allowed to build a three-dimensional (3-D) graphic in which the dependent variable Y was represented by a curvature surface as a function of X_i . The relationship between the response and independent variables can be directly visualized from the response surface plot.

The statistical software Design Expert v.8.0.1 (Minneapolis, USA) was used to establish the experimental design and the regression analysis of the experimental data (Table 2). The goodness of fit of the polynomial model equation was expressed by coefficient of determination R^2 . For the estimated coefficients, it is also possible to calculate Student's t-statistics and associated P values. Experiments were performed in triplicate, and mean values were determined.

Statistical analysis of data

The recombinant cyprosin B activity was represented by a second-order polynomial equation. The software Design Expert 8.0.1 (Minneapolis, USA) was applied to resolve the regression equation for determination of the maximum cyprosin B activity and the optimal concentrations for each variable.

Results and discussion

Optimization of culture medium using statistical analysis the experimental results showed that this mathematical tool can satisfactorily explain the effects of the variables concentration (glucose, YE and bactopectone) in the recombinant cyprosin B produced by *S. cerevisiae* BJ1991 strain in shake-flask cultures and in fermenter. The expression and secretion of the recombinant cyprosin B depend on the inducer concentration (galactose), which works also as a carbon source [3]. The maximum enzymatic activity was obtained for the concentration of galactose (40 g L^{-1}) in YPGal medium, which has shown to be a straightforward system for regulating and controlling expression of the CYP11 gene. Studies performed using the *S. cerevisiae* BJ1991 strain obtained the similar cyprosin B activity with 20 g L^{-1} of galactose (data not shown). As galactose is more expensive than the other components, it was possible to carry out the optimization study using the RSM to determine the optimal concentration of culture medium components and, consequently, to enhance cyprosin B production by transformed *S. cerevisiae* BJ1991 strain. For optimization using RSM, the variables (YE, bactopectone and glucose) were considered. To search for the optimum combination of these components in the culture medium, a CCD experimental plan was carried out (Table 2). The results of the second-order response surface model fitting in the form of analysis of variance (ANOVA) are given in Table 3.

Table 3 Analysis of variance (ANOVA) for the selected model ANOVA

Source	SS	df	MS	Fisher test	P value
Regression	140382.6	9	15598.1	7.36	0.0123
Residual	12716.8	6	2119.5		
Total	153099.4	15			

SS Sum of Squares, df degrees of freedom, MS mean squares

The ANOVA analysis, through the quadratic regression model, revealed that the second-order response surface model obtained is highly significant, as it is evident from the high Fisher's test ($F_{\text{model}} = \text{mean square regression} / \text{mean square residual} = 7.36$) and the low probability value ($P > 0.0123$). A F-test value (7.36) higher than the tabled value $F_{(9, 6)} = 2.96$ for 10% significance level representing a significative model. F-test for regression was significant at a level of 10% ($P < 0.1$), indicating that the model is appropriate and can adequately explain the variation observed in enzyme biosynthesis with the designed levels of the factors. The R^2 coefficient obtained, of 0.92, suggests that it is a reliable model and that 92% of the sample variation of cyprosin B production is attributed to a high correlation between the independent variables. Only 8% of the total variation of cyprosin B production is not explained by the model. The relatively high-adjusted determination coefficient ($R^2_{\text{Adj}} = 0.79$) accounts for a high significance of the model [29]. The coefficient of variation (CV) indicates the degree of precision with which the calculations are compared. Usually, a high CV value represents low reliability of the experiment. The low value obtained ($CV = 13.8\%$) indicates a small residual variability data and the greater reliability of the experiments performed [30].

The P values are used for checking the significance of each coefficient which, in turn, it is necessary to understand the pattern of the mutual interactions between the important variables, where the small magnitude of P values means that the corresponding coefficients are more significant. A positive coefficient represents an effect that favors the optimization of cyprosin B production, while a negative value indicates an antagonistic effect.

The three variables (glucose, YE and bactopectone), according to the analysis of Table 4, influencing positively the cyprosin B biosynthesis, i.e., inside the concentration range evaluated, raised values implied an increase in enzyme biosynthesis, which showed high significance on regression coefficients with the low P value < 0.1 ($P_{X1} = 0.0022$; $P_{X2} = 0.0059$ and $P_{X3} = 0.0454$; $P_{X13} = 0.0759$; $P_{X3,2} = 0.0684$). The glucose had more influence than yeast extract and bactopectone, which proof that yeast has more affinity for glucose for metabolic growth. This affinity for glucose proves that sugar is easily metabolizable by the yeast *S. cerevisiae* because it directly enters the glycolytic pathway as glucose-6-phosphate by the specific transporters [31]. The bactopectone and the yeast extract, as nitrogen sources, are constituted by essential nutrients for the yeast cell growth and function as the precursors of biomolecules that are fundamentals to the development of the microorganism [32].

The bactopeptone had too high significance at the quadratic level $P_{X_3^2}=0.0684$. According to the corresponding regression coefficient, (-33.5) showed a negative effect on enhancing cyprosin B activity, which means that the raise of quadratic directly influenced the decrease in recombinant cyprosin B production. The interaction between glucose and bactopeptone ($P_{X_1X_3} = 0.0759$) had too high significance over cyprosin B biosynthesis. Relative to regression coefficient, (34.8) showed a positive influence over cyprosin B production. According to this statistical analysis, the variables studied can act as limiting nutrients, and a slight variation registered in their concentration induces small changes over recombinant cyprosin B production.

Table 4 The least squares fit and parameter estimates (significance of regression coefficient)

Model term	Regression coefficient	Standard error	<i>t</i> (1)	<i>P</i> value
Mean interaction	353.9	32.49	10.900	0.0123
X_1	63.8	12.47	5.107	0.0022
X_1^2	-6.7	15.14	-0.453	0.6745
X_2	52.0	52.05	12.473	0.0059
X_2^2	16.1	15.14	1.059	0.3273
X_3	31.4	12.47	2.515	0.0454
X_3^2	-33.5	15.14	-2.221	0.0684
X_1X_2	-3.4	16.30	-0.208	0.8426
X_1X_3	34.8	16.30	2.134	0.0759
X_2X_3	-22.1	16.30	-1.357	0.2229

Analysis of surface plots

The empirical relationship between recombinant cyprosin B production (Y) and the experimental variables obtained by the application of the RSM is represented mathematically by the following equation (Eq. 3):

$$\begin{aligned} Y = & 353.87 + 63.76X_1 + 52.00X_2 + 31.37X_3 \\ & - 3.38X_1X_2 + 34.88X_1X_3 - 22.13X_2X_3 \\ & - 6.67X_1^2 + 16.13X_2^2 - 33.54X_3^2 \end{aligned} \quad (3)$$

where Y is the response for the recombinant cyprosin B activity; X_1 , X_2 and X_3 represent the variables glucose, YE and bactopectone, respectively.

The regression equation was represented in 3-D response surface plots, and each contour curve corresponds to an infinite number of combinations between two test variables, while the third is maintained in optimal concentration. The yield for different concentrations of the variables can also be predicated from the respective response surface plots (Fig. 1a–c). The maximum predicted yield is indicated by the surface confined in the response surface diagram.

The response surface from YE–glucose interaction plot (Fig. 1a) revealed that the increase in the recombinant cyprosin B activity is related to high concentration of YE (15 g L⁻¹) and glucose (30 g L⁻¹). For the bactopectone– glucose interaction plot (Fig. 1b), the high cyprosin B activity is related to the range of optimal concentrations of bactopectone (15–30 g L⁻¹) and of glucose (20 and 30 g L⁻¹). The bactopectone–YE interaction plot (Fig. 1c), which shows high cyprosin B activity, was achieved for the 10–15 g L⁻¹ for YE, and for bactopectone concentrations between 20 and 30 g L⁻¹. These data confirmed that the optimal values for each variable were within concentration range that corresponds to high cyprosin B activity. In general, cyprosin B production increases with high glucose concentration.

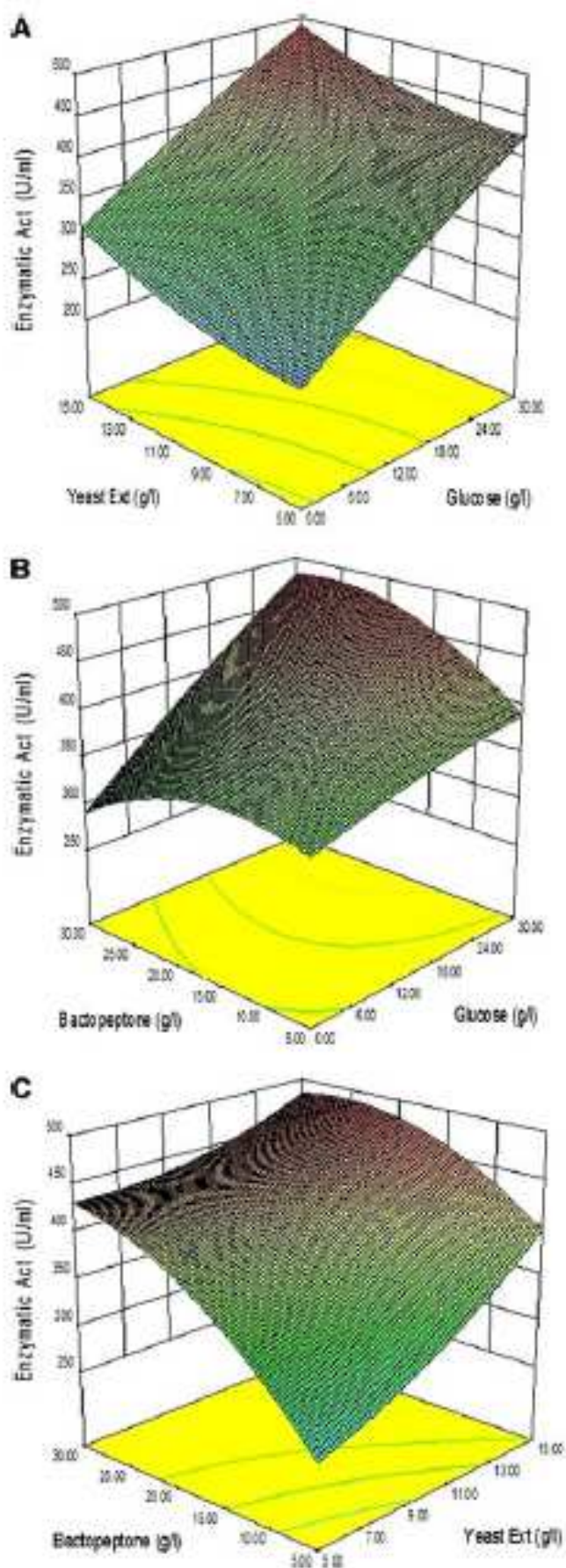


Fig. 1 Plots of response surface of recombinant cyprosin B activity with the fixed parameters: **a** bactopeptone; **b** YE and **c** glucose concentrations at their optimum points

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The regression equation, maximized by the Design Expert v.8.0.1 (Minneapolis, USA) software, allowed to obtain the following optimal coded units for test variables: $X_1 = 0.97$; $X_2 = 1.00$ and $X_3 = 0.75$, which corresponds to optimum concentrations of 30 g L⁻¹ glucose, 15 g L⁻¹ YE and 27 g L⁻¹ bactopectone, for the maximal recombinant cyprosin B activity of the 488 U mL⁻¹, predicted by the mathematical model with a high model accuracy (90%).

Validation experiments were carried out to verify the accuracy of the models, and the results showed that the predicted values are according to the experimental results. The model validation was carried out in shake-flask culture, and using the optimal concentrations of culture medium components has resulted in a cyprosin B activity of 519 U mL⁻¹, representing an increase of 46%, comparatively with the original medium (357 U mL⁻¹) and an increase of the 16% for biomass.

Bioreactor cultivation

The predicted optimal medium composition was applied to *S. cerevisiae* BJ1991 cultivation in a 5-L bench fermenter for the production of recombinant cyprosin B during which the pH and dissolved oxygen concentration were controlled and monitored in situ. For the optimal culture medium, a 17 g dcw L⁻¹ biomass was obtained, which represents a 82% increase and 2769 U m⁻¹ cyprosin B activity, meaning an increment of 139% compared to the original fermentation medium or to the culture in bioreactor of *S. cerevisiae* W303-1A strain [3] (Fig. 2). The secretion rate of active cyprosin B increased 11% probably due to the culture medium optimization that would have resulted in an improvement in the culture medium performance for enzyme production [33]. This positive evolution achieved may be related to the appropriate growth conditions created inside the bioreactor, such as adequate stirring and homogenization of culture medium, pH and temperature control, but mainly high dissolved oxygen concentration, which promotes cells growth and, consequently, the enzyme biosynthesis [30, 34]. The availability of high amounts of amino acids, co-factors and other non-nutritional trace components present in the bactopectone and in the YE that are essential for improving cell growth, yeast survival, protein expression and high cell density may also be responsible for a high metabolic rate, leading to an increased expression of recombinant cyprosin B and, consequently, its secretion to the culture medium.

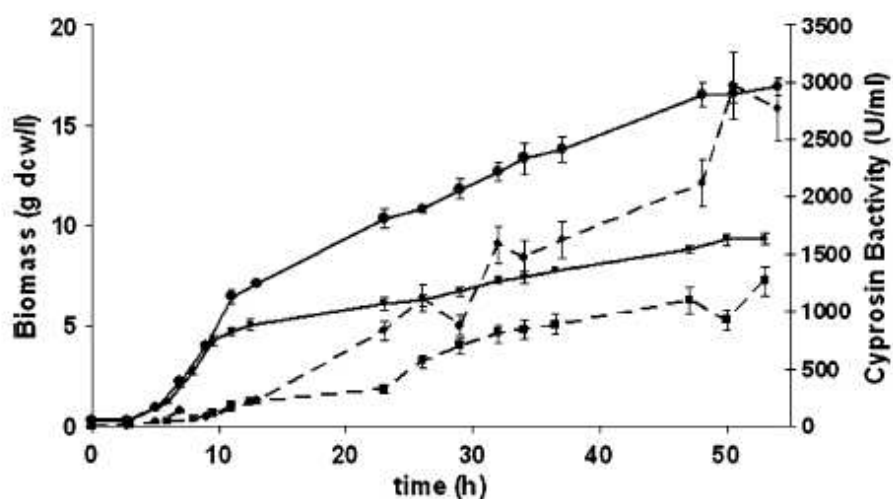


Fig. 2 Evaluation of *Saccharomyces cerevisiae* BJ1991 strain during cell growth in bioreactor using the optimized culture medium: *Full line and circles*–Biomass under optimized conditions; *Full line and squares*–Biomass in non-optimized conditions (control); *Dash line and circles*–Cyprosin B activity under optimized conditions; *Dash line and squares*–Cyprosin B activity under non-optimized conditions (control)

The recombinant cyprosin B presents milk-clotting ability similar to cyprosin B from *Cynara cardunculus* flowers (Fig. 3a and b). The increase in milk-clotting efficiency may result from the increased production of recombinant cyprosin B obtained by culturing the *S. cerevisiae* strain in the culture medium optimized according to the results obtained in this study. It is thus realistic to consider that taking into account the results here described, recombinant cyprosin B can now be produced on a large-scale and may constitute a new and effective alternative for cheese-making enzymes of plant origin.

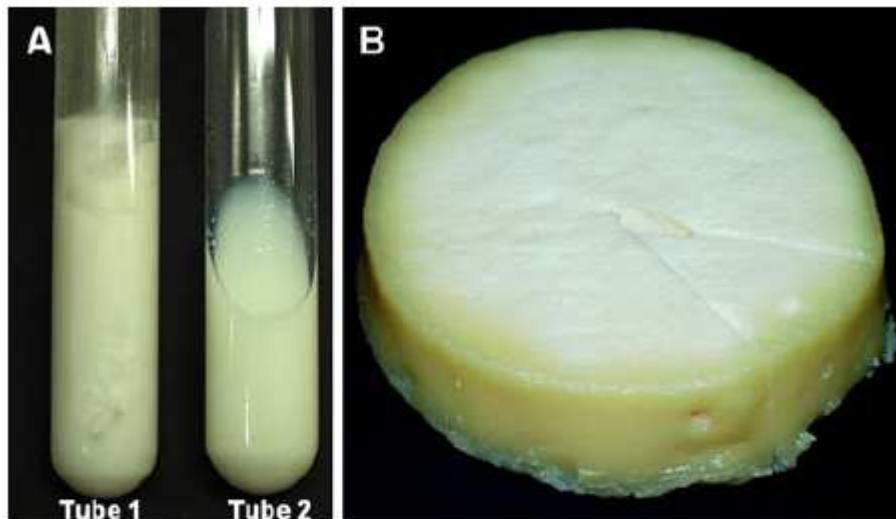


Fig. 3 a Milk clotting using samples of recombinant cyprosin B obtained after fermentation using BJ1991 pCAF15 strain (Tube 2) comparatively with the control (Tube 1) without adding recombinant cyprosin B. No curd was formed. **b** Cheese made with recombinant cyprosin B, presenting high clotting activity, produced in optimized culture medium

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