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Kluyveromyces lactis β-galactosidase crystallization using full-factorial experimental design

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Abstract

Kluyveromyces lactis β-galactosidase is an enzyme with numerous applications in the environmental, food and biotechnological industries. Despite of its biotechnological interest, its three-dimensional structure has not yet been determined. The growth of suitable crystals is an essential step in the structure determination of a protein by X-ray crystallography. At present, crystals are mostly grown using trial-and-error procedures since their growth often depends on the combination of many different factors. Testing the influence on crystallization of even only a small number of these factors requires many experimental set-ups and large amounts of protein. In the present work, a full-factorial design has been used in order to find conditions for obtaining good-quality crystals of *K. lactis* β-galactosidase. With this full-factorial method protein crystals have been obtained.

Keywords: β-Galactosidase; Full-factorial design; *Kluyveromyces lactis*; Purification; Crystallization

1. Introduction

The microbial lactase or β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) from the yeast *Kluyveromyces lactis*, the enzyme which is responsible for the hydrolysis of lactose into glucose and galactose, has outstanding biotechnological interest. Therefore it has attracted the attention of researchers and industries because of its important applications in the fields of medicine (treatment of lactose intolerance), food technology (to prevent lactose crystallization and increase its sweetening power) and the environment (cheese whey utilization). Although much of the work on *K. lactis* β-galactosidase has dealt with the production [1–4], the use [5] and biochemical characterization [6–8], to the best of our knowledge, very little has been reported about its structure [8].

The growth of suitable protein crystals is an essential step in the structure determination of a protein by X-ray crystallography. At present, crystals are mostly grown using trial-and-error procedures, whereby various factors: pH, temperature, salt concentration, etc., are systematically varied until crystals are obtained. Usually, in these experiments, the different factors are varied only over a narrow range of values. The use of this method often requires large amounts of material and is frequently time consuming.

Carter and Carter [9] demonstrated that the application of a full-factorial approach to protein crystallization could reduce substantially the number of crystallization trials. Their method, which is based on a factorial approach to experimental design [10], permits the assay of a large number of crystallization conditions with as few experiments as possible. This is accomplished by varying more than one factor at a time in a given experiment; this saves material, and from the analysis of the results, it is possible to readily determine the factors that are critical for crystallization. We present here the details of a full-factorial design to find conditions for growing good-quality crystals of *K. lactis* β-galactosidase.

2. Experimental

2.1. Strains and culture conditions

The following strains were used: *Kluyveromyces lactis* NRRL-Y1140 (*MATa*, wild type) and *Saccharomyces cerevisiae*

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BJ3505 (*pep4::HIS3*, *prb-Δ1.6R HIS3*, *lys2-208*, *trp1-Δ101*, *ura 3-52*, *gal2*, *can1*). The BJ3505 strain was purchased from Eastman Kodak.

Liquid batch cultures of wild type and transformed cells were grown in Erlenmeyer flasks filled with 20% volume of culture medium at 250 rpm and 30 °C, unless otherwise stated. *K. lactis* wild type cells were growth in YPL (1% yeast extract, 0.5% bactopeptone, 0.5% lactose) whereas transformed *S. cerevisiae* BJ3505 cells were growth in YPHSM (1% yeast extract, 8% bactopetone, 1% dextrose, 3% glycerol, 20 mM $CaCl_2$). In the late case, as inocula, a suitable volume of a stationary phase culture in complete medium (CM) [11] without the corresponding auxotrophic amino acid was added to obtain an initial OD_{600} of 0.2. Samples were taken at regular time intervals to measure growth (OD_{600}) and intracellular β-galactosidase activity.

2.2. Vectors

The YEpFLAG1-LAC4 [2] containing the *LAC4* gene, which codes for *K. lactis* β-galactosidase, inserted between the yeast *ADH2* promoter and *CYC1* terminator was used. This plasmid also contains the sequence of the FLAG peptide for the immunological detection and affinity purification of the FLAG fusion protein.

2.3. Molecular biology procedures

Yeast strains were transformed using the lithium acetate procedure [12]. Plasmid uptake and β-galactosidase production by the transformed strains were identified on plates with the chromogenic substrate X-gal in the corresponding auxotrophic medium.

2.4. β-Galactosidase activity assays and protein determinations

The method of Guarente [13] as previously described [2] was used. One enzyme unit (EU) was defined as the quantity of enzyme that catalyzes the liberation of 1 μmol of *ortho*-nitrophenol from *ortho*-nitrophenyl-β-D-galactopyranoside per min under assay conditions.

Protein was determined by the method of Bradford [14] using bovine serum albumin (Sigma) as a standard.

2.5. Preparation of crude protein extracts

Crude protein extract was prepared as described previously [7] from cells cultured in 1 l of YPL or YPHSM up to an A_{600nm} of 2 (about 3 mg dry wt/ml).

2.6. Purification of β-galactosidase

The purification of β-galactosidase from a crude protein extract of the strain of *K. lactis* NRRL-Y1140 and from a YEpFLAG1-LAC4 transformed *S. cerevisiae* BJ3505 strain was performed using different chromatographical techniques.

In the first trial, a column with 5 ml agarose-*p*-aminopheyl-β-D-thiogalactoside (Sigma Chemical, USA) was equilibrated with 50 mM phosphate buffer pH 7, and the enzyme was eluted with 0.1 M sodium borate, pH 10. Fractions of 1 ml were collected at a flow rate of 100 μl/min. The pH of the collected fractions was neutralized to avoid denaturation.

In the second purification method assayed, a column with 0.2 ml of ANTI-FLAG M2 affinity gel (Sigma Chemical, USA), useful for purification of FLAG fusion proteins, was equilibrated with TBS (150 mM NaCl, 50 mM Tris–HCl, pH 7.4) and the elution of the bound FLAG fusion protein was by competition with a solution containing 100 μg/ml FLAG peptide (Sigma Chemical, USA).

All purification steps were carried out at 4 °C. The β-galactosidase activity was assayed in the eluted fractions obtained from chromatographic steps. Active fractions were pooled and, when required, concentrated by filtration in Amicon ULTRA-4 (Millipore, UFC 803024).

2.7. Polyacrylamide gel electrophoresis

This was performed as described in Becerra et al. [1].

2.8. Protein crystallization

Crystals were grown at several conditions at 20 °C by vapour diffusion in hanging drops containing 1–3 μl of protein solution (9 mg/ml) and 1 μl of reservoir solution.

2.9. Experimental design and statistical data analysis

Factorial experimental design was created and data were analyzed with the aid of version 5.1 of the *STATGRAPHICS Plus* software for *Windows* (*Statistical Graphics Corporation*).

The statistical significance of differences between means was determined by Student's *t*-test performed with the same software. *P*-values <0.05 were considered significant.

3. Results and discussion

3.1. Purification of K. lactis β-galactosidase

The success of the crystallization process starts at the protein purification level. In a previous work, the expression and purification of *K. lactis* β-galactosidase in *E. coli* as a His-tagged recombinant enzyme was tried but left out, due to the formation of insoluble inclusion bodies and the irreversible inhibitory effect of imidazole on the enzyme [15]. In our study, *K. lactis* β-galactosidase purification was achieved by two different procedures: affinity chromatography on agarose-*p*-aminophenyl-β-D-thiogalactoside and immunoaffinity on ANTI-FLAG M2 affinity gel.

The purification of the enzyme from a crude protein extract of the *K. lactis* strain NRRL-Y1140 by affinity chromatography resulted in a purification factor of 2.7 over the crude extract, having an overall yield based on total enzyme units of 17.4%. Values are comparable to those previously reported for

Table 1
Summary of the purification procedure of *K. lactis* β-galactosidase by affinity chromatography (A) and *of K. lactis* β-galactosidase fused to the FLAG peptide by immunoaffinity (B)

	Step	Total protein (mg)	Total EU	Yield (%)	Specific activity (EU/mg)	Purification factor
A	Crude extract	87	137 250	100	1577.59	1
	Affinity and microultrafiltration	1.79	23 882	17.4	4259.5	2.7
B	Crude extract	133.10	104 610	100	785.95	1
	Immunoaffinity chromatography	1.91	71 980	68.81	37705.61	47.98

the same enzyme [7]. The results of this purification process are summarized in Table 1A. *K. lactis* β-galactosidase was also purified from a *S. cerevisiae* BJ3505 strain transformed with YEpFLAG1-LAC4 by affinity purification of the FLAG fusion protein. In this case, a 1.43% protein recovery with a yield of 68.8% and an increase in specific activity of 47.98-fold was obtained (Table 1B).

The homogeneity of the isolated β-galactosidases was examined by SDS-PAGE of the purified enzyme (Fig. 1), both preparations show a main protein band with the approximate molecular weight of 124 kDa that agreed with the one predicted from the sequence of *LAC4*, the unique gene coding for β-galactosidase present in the *K. lactis* genome [16].

These data demonstrate the usefulness of both tested purification procedures for obtaining *K. lactis* β-galactosidase protein. Although the second procedure gave the highest purification factor and turned out to be more effective.

3.2. Initial crystallization screens

Crystallization of macromolecules is usually performed by a somewhat organized trial-and-error procedure using available kits. However, use of these kits locks the experimenter to a relatively narrow set of historical conditions. In our case, the search strategy to get optimal crystallization of *K. lactis* β-galactosidase was based on the use of conditions that have already rendered useful for obtaining crystals of a homologous

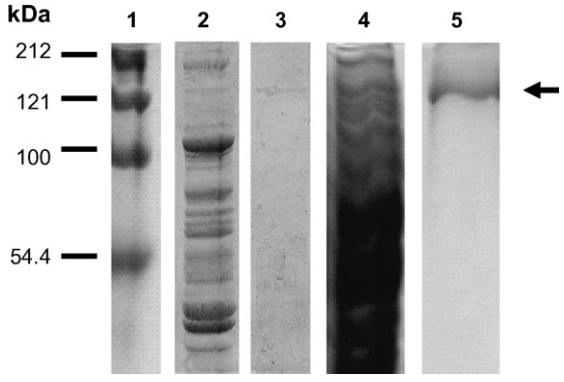

Fig. 1. SDS-PAGE of purified *K. lactis* β-galactosidase. Lane 1, molecular weight markers; lane 2, 60 μg of a crude extract from *K. lactis* NRRL-Y1140; lane 3, 1.5 μg of *K. lactis* β-galactosidase purified by affinity chromatography from *K. lactis* NRRL-Y1140, lane 4, 90 μg of a crude extract of *S. cerevisiae* BJ3505 transformed with YEpFLAG1-LAC4; lane 5, 3.5 μg of *K. lactis* β-galactosidase purified by immunoaffinity. β-Galactosidase is indicated by an arrow.

protein with similar size and function, the β-galactosidase from *E. coli* [17]. Reproducing these conditions, small protein crystals were obtained in presence of 0.1 M Tris pH 8.0 and different concentrations of $(NH_4)_2SO_4$ (0.02 and 0.2 M) and PEG 6000 (5% and 10%) (Fig. 2A).

3.3. Optimization of the crystallization conditions

The formation of crystals depends on the concentration of macromolecule and precipitant. At higher concentrations of macromolecule, less precipitant is required for crystallization. The function of the different precipitants such as polyethylene glycol and ammonium sulphate in the crystallization drop is to alter the protein–solvent or protein–protein contacts so that the protein molecules precipitate out of solution, preferably as ordered crystals and not as disordered aggregates. In our case, in order to identify optimal conditions for crystal growth, including crystal volume and shape improvements, we studied, by means of a full-factorial design, the influence of three variables (% PEG 6000, $(NH_4)_2SO_4$ and protein concentrations), and their interactions on the response. The range and coding criteria of the variables used are given in Tables 2 and 3 shows the experimental matrix and the results obtained for the analyzed response, the quality of crystals. The quality obtained was quantified taking into account three parameters: morphology, size and amount of crystals. A score of 2 points was given to crystals with regular morphology and 1 point to crystals with irregular morphology. In the center of the experimental domain (0 coded value to the three variables), crystals showed a similar size, and therefore were considered as average size and scored with 2 points. Bigger crystals than those obtained in the center of domain were scored with 3 points, and smaller crystals with

Table 2
Experimental domain and codification of the variables used in the full-factorial design

Natural values			
Coded values	PEG 6000 (P, %)	$(NH_4)_2SO_4$ (A, M)	Protein concentration (PRO, μg)
−1	5	0.02	10
0	10	0.1	15
+1	15	0.18	20

Codification: $V_c = (V_n - V_0)/DV_n$; decodification: $V_n = V_0 + (\Delta V_n \times V_c)$ where V_c is the coded value, V_n is the natural value, V_0 is the natural value in the center of the experimental domain and ΔV_n is the increase in the natural value corresponding to 1 U of growth in the coded value.

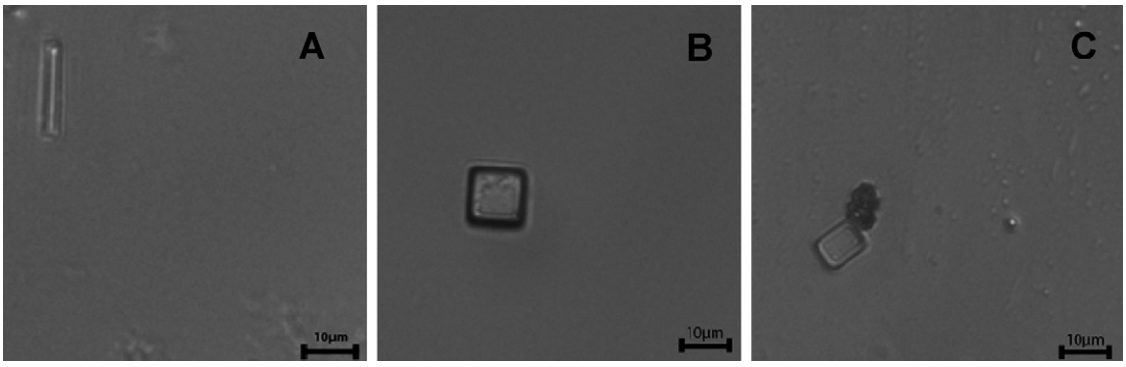

Fig. 2. Photography of one *K. lactis* β-galactosidase crystal obtained in presence of 0.1 M Tris, pH 8.0, 0.02 M $(NH_4)_2SO_4$ and 5% PEG 6000 (A). Protein crystals obtained using the full-factorial design approach (B and C). Photographs were taken with the objective 40×.

1 point. Finally, 1 point was given to conditions which showed multiple crystals, and two points were given to conditions with few crystals.

The ANOVA table (Table 4A) divides the variability in the response into separate pieces for each of the effects. It then tests the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. In this case, three effects have *P*-values less than 0.05 (with asterisk in Table 4A), indicating that they are significantly different from zero at the 95.0% confidence level.

After removing the no significant coefficients, the *P*-value for lack-of-fit in the ANOVA table (Table 4B) is greater to 0.05 (0.1149) and the model appears to be adequate for the observed data at the 95.0% confidence level. The lack of fit test is designed to determine whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate settings of the factors.

The R^2 statistic indicates that the model, as fitted, explains 91.7017% of the variability in the response. The adjusted R^2 statistic, which is more suitable for comparing models with different numbers of independent variables, is 88.5898%. The standard error of the estimate shows the standard deviation of the residuals to be 0.64145. The mean absolute error (MAE) of 0.423611 is the average value of the residuals.

The Durbin–Watson (DW) statistic tests the residuals to determine if there is any significant correlation based on the order in which they occur in the data file. Since the DW value is greater than 1.4 (2.05063), there is probably not any serious autocorrelation in the residuals.

The system can be represented by the following codified equation (significance tested by Fisher *F*-test) in which only the concentration of protein, the interaction between the % PEG 6000 and ammonium sulphate concentration and the interaction between the three variables present influence in the response:

$$\text{Quality of crystals} = 3.83333 + 1.375 \times \text{PRO} - 0.875 \times P \times A + 1.375 \times P \times A \times \text{PRO}$$

Table 3
Experimental results of the full-factorial design (2^3) for the study of *K. lactis* β-galactosidase crystals quality obtained taking into account three parameters: morphology, size and amount of crystals

	P	*A*	PRO	Quality of crystals
1	1	1	1	6
2	−1	1	1	4
3	1	1	−1	0
4	−1	1	−1	5
5	1	−1	1	5
6	−1	−1	1	5
7	1	−1	−1	4
8	−1	−1	−1	0
9	0	0	0	5
10	0	0	0	4
11	0	0	0	4
12	0	0	0	4

Variables are according to Table 2.

Table 4
Analysis of variance for the response (quality of the crystals) in the full-factorial design (2^3) studied before (A) and after (B) removing the no significant coefficients and analysis of the significance and adequacy of the proposed model

	Source	Sum of squares	Degree of freedom	Mean square	*F*-ratio	*P*-value
A	*P*:PEG	0.125	1	0.125	0.50	0.5305
	A:ammonium	0.125	1	0.125	0.50	0.5305
	PRO:protein	15.125	1	15.1250	60.50	0.0044*
	$P \times A$	6.125	1	6.125	24.50	0.0158*
	$P \times$ PRO	1.1250	1	1.125	4.50	0.1240
	$A \times$ PRO	0.125	1	0.125	0.50	0.5305
	$P \times A \times$ PRO	15.1250	1	15.1250	60.50	0.0044*
	Lack-of-fit	1.04167	1	1.04167	4.17	0.1339
	Pure error	0.75	3	0.25		
	Total (corr.)	39.6667	11			
B	PRO:protein	15.1250	1	15.1250	47.06	0.0002
	$P \times A$	6.1250	1	6.1250	19.06	0.0033
	$P \times A \times$ PRO	15.1250	1	15.1250	47.06	0.0002
	Lack-of-fit	1.04167	1	1.04167	3.24	0.1149
	Pure error	2.25	7	0.321429		
	Total (corr.)	39.6667	11			

(*) Significant coefficients. Variables according to Table 2. $R^2 = 91.7017\%$; R^2 (adjusted for d.f.) = 88.5898%; standard error of est. = 0.64145; mean absolute error = 0.423611; Durbin–Watson statistic = 2.05063 ($P = 0.4610$).

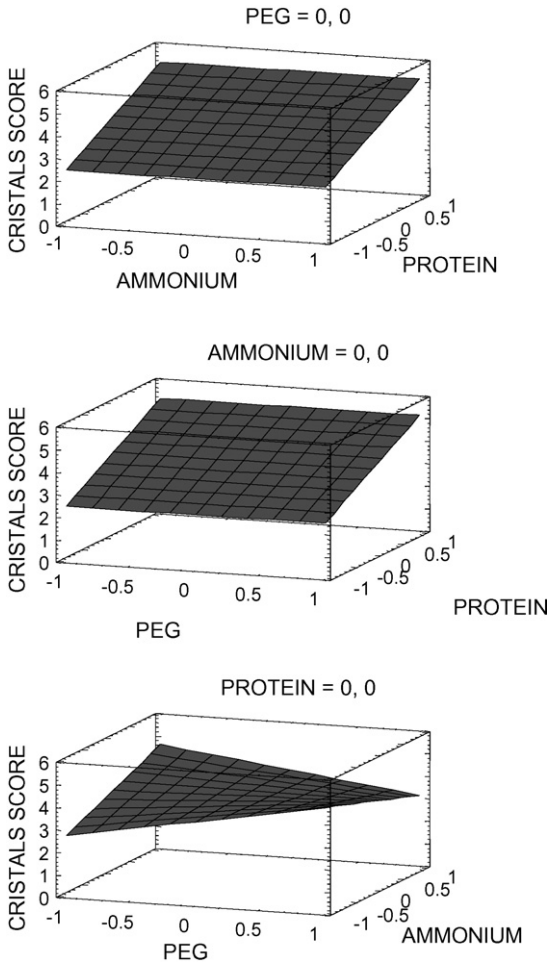

Fig. 3. Response surfaces obtained to identify optimal conditions for *K. lactis* β-galactosidase crystal growth according to the experimental plan defined in Table 2. Crystals score = response (quality of crystals taking into account: morphology, size and amount of crystals). Variable values and nomenclature can be seen in Table 2.

Some of the more representative surface responses corresponding to the mentioned equation are represented in Fig. 3. These surfaces are planes defined by pairs of variables having the third variable fixed (values −1, +1). As can be seen in Fig. 3, the response increases in each situation of our experimental domain when the concentration of the protein is increased (positive coefficient). The effect of % PEG 6000 and ammonium sulphate concentration, for the same protein concentration, is more complex because response increases in the corners, high % PEG 6000 and small ammonium sulphate concentration and vice versa small % PEG 6000 and high ammonium sulphate concentration. Therefore, the highest values of the response are obtained in the corners: $P = +1$, $A = -1$, PRO = +1 or $P = -1$, $A = +1$, PRO = +1.

Some of the *K. lactis* β-galactosidase crystals obtained with the optimal conditions obtained by this approach are shown in Fig. 2B and C.

4. Conclusions

A methodical and efficient approach has been carried out to growth *K. lactis* β-galactosidase crystals. The full-factorial screen with response surface optimization allowed us to find conditions for growing good-quality crystals with a small number of experiments to be performed. Optimal crystallization conditions for 20 μg of *K. lactis* β-galactosidase were obtained in the presence of 0.1 M Tris–HCl, pH 8, 15% PEG 6000 and 0.02 M $(NH_4)_2SO_4$. Advantages obtained in this approach include improvements in β-galactosidase crystal volume and shape and also in reproducibility. Similar designs could be of interest to get crystals from other proteins which have special difficulties to solve.

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References

[1] M. Becerra, E. Cerdán, M.I. González Siso, Biochim. Biophys. Acta 1335 (1997) 235–241.
[2] M. Becerra, S. Díaz-Prado, M.I. González Siso, E. Cerdán, Prot. Eng. 14 (2001) 379–386.
[3] M. Becerra, S. Díaz-Prado, E. Rodríguez-Belmonte, E. Cerdán, M.I. González Siso, Biotechnol. Lett. 24 (2002) 1391–1396.
[4] M. Becerra, E. Rodríguez-Belmonte, E. Cerdán, M.I. González Siso, J. Biotechnol. 109 (2004) 131–137.
[5] M. Becerra, E. Cerdán, M.I. González Siso, Recent. Res. Dev. Biochem. 4 (2003) 549–559.
[6] V. Athès, R. Lange, D. Combes, Eur. J. Biochem. 255 (1998) 206–212.
[7] M. Becerra, E. Cerdán, M.I. González Siso, Biotechnol. Tech. 12 (1998) 253–256.
[8] S.R. Tello-Solís, J. Jiménez-Guzmán, C. Sarabia-Leos, L. Gómez-Ruíz, A.E. Cruz-Guerrero, G.M. Rodríguez-Serrano, M. García-Garibay, J. Agric. Food Chem. 53 (2005) 10200–10204.
[9] C.W. Carter Jr., C.W. Carter, J. Biol. Chem. 254 (1979) 12219–12223.
[10] R.A. Fisher, The Design of Experiments, third ed., Oliver and Boyd, London, 1942.
[11] C.V. Lowry, J.L. Weiss, A. Walthall, R.S. Zitomer, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 151–155.
[12] H. Ito, Y. Fukuda, K. Murata, A. Kimura, J. Bacteriol. 153 (1983) 163–168.
[13] L. Guarente, Meth. Enzymol. 101 (1983) 181–191.
[14] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[15] C.S. Kim, E.-S. Ji, D.-K. Oh, Biotechnol. Lett. 25 (2003) 1769–1774.
[16] O. Poch, H. LǐHote, V. Dallery, F. Debeaux, R. Fleer, R. Sodoyer, Gene 118 (1992) 55–63.
[17] D.H. Juers, S. Hakda, B.W. Matthews, R.E. Huber, Biochemistry 42 (2003) 13505–13511.