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Extraction of Intracellular Proteins from *Kluyveromyces lactis*

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Summary

Several preparation methods of crude extracts of yeasts were compared in order to obtain a good recovery of intracellular proteins and a sample quality suitable for further chromatographic analysis as exemplified by β-galactosidase or lactase. Sonication was found to be the most effective method and the physiological state of the cells and growth conditions at the time of extraction are essential parameters for obtaining good yield. The procedure described is simple, rapid and applicable to other enzymes of interest.

Key words: β-galactosidase, purification, extraction, sonication, *Kluyveromyces lactis*

Introduction

The growing use of fermentation processes to manufacture specific products, including diverse heterologous proteins, enzymes or pharmaceuticals, is creating an increased demand for efficient systems for the recovery of intracellular materials from microorganisms.

Cell disruption is the first stage in the procedure of isolating intracellular materials (*1*). It constitutes an essential step in downstream processing, as it has considerable influence not only on the total quantity of the desired protein recovered, but also on its biological activity, its association with other cellular components, and the possible presence of proteolytic degradation and contaminants that may influence the subsequent purification steps (*2*).

A variety of methods can be used to break up cells, each one having its advantages and disadvantages. In this paper we have compared the efficiency of several methods for the preparation of crude extracts from the yeast *Kluyveromyces lactis* in order to obtain a good recovery of intracellular proteins and a sample quality suitable for further chromatographic analysis.

The crude extracts obtained by sonication resulted in the best yield and were used as protein mixtures in several microscale chromatography-based systems designed for β-galactosidase purification. β-galactosidase (β-D-galactoside-galactohydrolase, EC. 3.2.1.23) or lactase, a cytoplasmic protein, was chosen as a sample protein because of easiness to assay its activity and its important applications in the dairy industry (*3–6*).

Materials and Methods

Microorganisms and culture conditions

Kluyveromyces lactis NRRL-Y1140 was used in the extraction. Cultures were aerobically grown in YPL (1 % yeast extract, 0.5 % peptone and 4 % lactose) and YPD (1 % yeast extract, 0.5 % peptone and 0.5 % dextrose) at 30 °C. Cells were harvested by centrifugation at 4000 ×*g*.

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The yeast pellet was washed with distilled water once and stored at –20 °C.

For transformation, *Escherichia coli* JM109 strain (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiD(lac-proAB) F´[traD36 proAB$^+$ lacI^q lacZDM15]*) with the plasmid pLX8 by means of the usual DNA recombinant techniques (*7*) was used. The pLX8 plasmid carries the *LAC4* promoter and gene, which codes for *K. lactis* β-galactosidase, and the *LAC12* gene which codes for *K. lactis* lactose permease.

Extraction with solvents

The harvested cells (2 g wet weight) were suspended in 20 mL of 0.5 M phosphate buffer (pH = 9.5). After the addition of 1 % chloroform the cell suspensions were incubated at 37 °C for 3 hours (*8*). During extraction, the activity of released β-galactosidase was determined after cells were removed by centrifugation for 5 min at 4000 ×*g*. This activity was compared to intracellular enzymatic activity.

In other instances, 1 g (wet weight) of cells was suspended in different 80 % solvents (ethanol, methanol, isopropanol and *t*-butanol) (*9*). The extraction took 90 min at 30 °C. The solvent-cell mixture was then diluted with 100 mL of 0.1 M potassium phosphate buffer, (pH = 6.6) and incubated for 21 hours at 30 °C. The extracted cells were removed from the enzyme-enriched buffer by centrifugation and the enzymatic activity of released β-galactosidase was determined. Results were expressed as the percentage of released activity in relation to intracellular activity.

Extraction with mechanical procedures

The cells were harvested at different cell densities by centrifugation at 4000 ×*g* for 5 min at 4 °C and washed once with distilled water. They were suspended in 1 mL of buffer (20 mM Tris-HCl, pH = 7.8, 300 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 10 % glycerol buffer with 0.1 mM PMSF (phenylmethylsulfonylfluoride), 4 mM pepstatin, 4 mM leupeptin and 2 μM β-mercaptoethanol) per gram wet weight and broken up using different techniques: *a)* stirred vigorously in a vortex for 20 sec and 20 sec on ice; *b)* using the same method but with the addition of 0.2 g of 1.5 mm glass beads; *c)* using a sonicator at 16 μm for 20 min at 4 °C at 5 min intervals after each 3–4 min exposure. In all cases cell-debris was removed by centrifugation at 48000 ×*g* for 90 min at 4 °C and the cell-free extract recovered in the supernatant.

Extraction with enzymes

The cells were suspended in 1.2 M sorbitol, 10 mM KH_2PO_4, pH=6.8, and 25 mM β-mercaptoethanol, washed twice and resuspended in 1.2 M sorbitol, 10 mM KH_2PO_4, pH=6.8 and 0.6 % lyticase (from *Arthrobacter luteus*, Sigma Chemical, USA, Ref.: L-8012). The mixture was incubated at 30 °C for 45-60 min and centrifuged at 2000 ×*g* at 4 °C for 5 min. The pellet was suspended in distilled water and after lysis the suspension was centrifuged at 2000 ×*g* for 5 min. β-galactosidase activity was measured in the supernatant which contains the cytoplasmic fraction (*10*).

β-galactosidase activity

To determine β-galactosidase activity, the Guarante's method (*11*) was used. Liberated *o*-nitrophenol (ONP) was measured spectrophotometrically at 420 nm (molar absorbance coefficient of 4500 L mol^{-1} cm^{-1}) (*12*).

One enzyme unit (E.U.) is defined as the quantity of enzyme that catalyzes the liberation of 1 μmol of ONP from *o*-nitrophenyl-β-D-galactopyranoside per min under assay conditions.

Protein determination

Protein concentration was determined according to the procedure of Bradford (*13*) using bovine serum albumin as the standard.

Purification of β-galactosidase

The purification of β-galactosidase was performed using several chromatographical techniques. All purification steps were carried out at 4 °C. The activity was assayed in the fractions obtained from chromatography. Active fractions were pooled and, when required, concentrated by filtration on Amicon Microcon-100 YM membranes.

Gel filtration chromatography

FPLC Smart system from Pharmacia was used. The column Superose 12 PC 3.2/30 (2.4 mL) pre-packed with Superose 12, a highly cross-linked, 12 % agarose-based medium, was equilibrated and further eluted with 50 mM sodium phosphate, pH=7.0, 0.15 M NaCl. Absorbance (280 nm) was measured on at line. The elution rate was 40 μL/min and the eluate was collected in 0.1 mL aliquots.

Ion-exchange chromatography

FPLC Smart system from Pharmacia was used. The column Mono Q PC 1.6/5 (0.10 mL) pre-packed with Mono Q (Quarternary amino ethyl) was equilibrated with 20 mM triethanolamine, pH=7.5. Proteins were eluted with a linear gradient of NaCl from 0.0 to 1.0 M in 20 min at a flow rate of 100 μL/min and the eluate was collected in 0.1 mL fractions.

Affinity chromatography

The column with 5 mL agarose-*p*-aminophenyl-β-D--thiogalactoside (Sigma Chemical, USA) was equilibrated with 50 mM phosphate buffer, and the enzyme was eluted with 0.1 M borate buffer, pH=10 (*14*). Aliquots of 1 mL were collected at a flow rate of 100 μL/min and pH was adjusted at 7 to avoid denaturation.

Results

A study of the extraction with solvents

The effect of 1 % chloroform extraction on β-galactosidase release from cells was studied at different times. Maximal extracellular activity was reached at 60 min and thereafter the activity began to fall sharply (Fig. 1). At this point the percentage of released activity in the

buffer in reference to the initial intracellular activity was approximately 1 %. As this percentage is very low, and several solvents were found to be able to release β-galactosidase from other yeasts (*9*), other compounds (methanol, ethanol, isopropanol and *t*-butanol) were used in order to improve the extraction.

The best results were obtained with methanol after 21 h of treatment and with ethanol and *t*-butanol after 5 h of contact with the extraction buffer (Table 1).

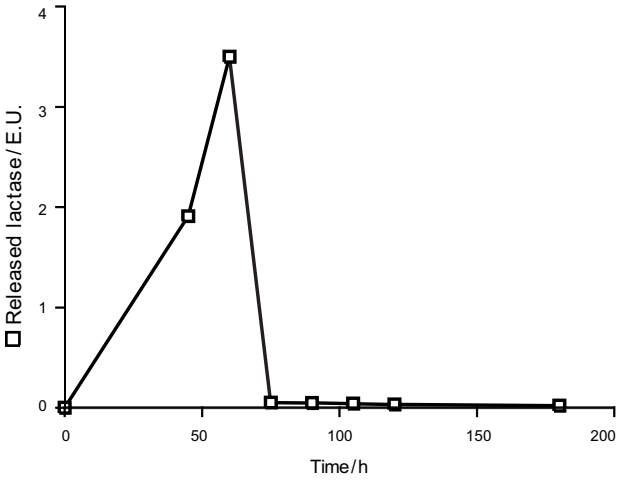

Fig. 1. Effect of incubation time with 1 % chloroform on lactase release; cells were suspended in 0.5 M phosphate buffer (pH = 9.5) with 1 % chloroform and incubated for 3 hours. Absolute values of lactase in the medium are given (in all cases SE<10 %)

Table 1. Effect of different solvents on lactase release expressed as the percentage of released activity in reference to intracellular activity (in all cases SE<10 %)

Solvent	5 hours	21 hours
t-Butanol	9.44	0.27
Ethanol	9.3	5.31
Methanol	1.74	15
Isopropanol	0.7	3.5

Cells were treated with 80 % solvent for 90 min. The solvent cell mixture was then diluted with 100 mL of 0.1 M potassium phosphate buffer, pH = 6.6 and incubated for 21 h at 30 °C. Samples were taken at 5th h and 21st h of extraction.

A study of the extraction with mechanical procedures

Different methods of mechanical cellular breakage were assayed. Extraction with vigorous stirring (vortex) resulted in a protein release of 0.12 ± 0.013 mg/mL (mean ± SE, n=11) with a β-galactosidase activity of 0.61 ± 0.079 E.U (mean ± SE, n=11). Percentage of released activity in reference to intracellular activity was about 1 ± 0.039 (mean ± SE, n=11). A similar result was obtained when cells were treated identically, but with 0.2 g of glass-beads. The best results were achieved with sonication, yielding about 50-fold more protein and about 9800-fold more activity than in the other two mechanical methods (Table 2). In this case the percentage of released activity reached 64 ± 4.8 (mean ± SE, n=5).

Table 2. Comparative effect on protein release of the different mechanical procedures used in this paper

Method	γ(protein)/(mg/mL) (mean ± SE)	activity (β-galactosidase) /E.U. (mean ± SE)
Vortex (n=11)	0.12 ± 0.013	0.61 ± 0.079
Glass-beads (n=2)	0.1 ± 0.028	0.95 ± 0.37
Sonication (n=5)	5.45 ± 1.07	7308 ± 2621
Sonication* (n=2)	57 ± 9.2	87 ± 26.3

* Results of sonication procedure over *E. coli* cells transformed with the pLX8 plasmid

Since bacteria are more sensitive to sonical disruption than yeasts, we transformed a deficient β-galactosidase *Escherichia coli* strain with the pLX8 plasmid. The plasmid carries the *LAC4* gene and promoter region, which codes for *K. lactis* β-galactosidase. The protein obtained by sonication of these transformants was 11 times higher than that obtained by yeast sonication; however, β-galactosidase activity was 84 times lower (Table 2). This is attributable to the fact that β-galactosidase heterologous production in bacteria is not as effective as natural production in yeast.

In order to improve the results obtained with the sonication procedure, extracts from *K. lactis* cells were prepared in different conditions. Cells grown until they reached diverse A_{600} values were sonicated and the amount of protein and β-galactosidase obtained measured. Both parameters appeared to be approximately proportional to the initial absorbance with a maximum of protein and enzymatic activity when cells were close to the value of 2 at A_{600}. Thereafter there was a decrease in the activity and protein recovery (*Fig. 2*).

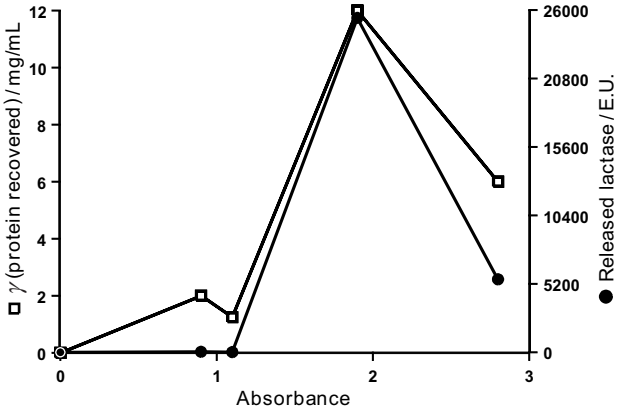

Fig. 2. Effect of the growth phase on protein release and lactase activity with the sonication procedure; cells were harvested at different A_{600} and broken using a sonicator (in all cases SE<10 %)

Using different culture volumes (from 500 to 1000 mL) at the same absorbance yielded similar results with

the difference being less than 4.5 %. Apparently the efficiency of sonication is not significantly influenced by cell density. The physiological state of the cells at the time of sonication seems to be a determining factor. During sonication the homogenate initially becomes more viscous as nucleic acids are released from the cells (*15*). The viscosity decreases as sonication is continued.

The enzymatic extraction procedure

Cells grown in two different media were used to study the enzymatic extraction procedure. The percentage of released activity compared to intracellular activity in cells growing in a medium with dextrose reached about 24 % (Fig. 3A), whereas for cells growing in a medium with a β-galactosidase inducer, lactose, the percentage was higher for about 61 % at the point of maximum recovery (Fig. 3B). In both cases, samples were taken in triplicate at the point of the maximum recovery yielding absolute average values of 23.5 ± 1.97 % (mean ± SE, n = 3) and 61.4 ± 16.72 % (mean ± SE, n = 3), respectively. As in the sonical extraction, cells harvested during the log phase of growth appear to be more sensitive to proteolytic degradation.

In addition to the high cost of the enzyme treatment, there is another important drawback: during incubation a loss of approximately 34 ± 1.54 % (mean ± SE, n = 12) of β-galactosidase activity took place.

Purification of β-galactosidase

With the crude protein extract obtained by sonication a process of purification was performed (*16*,*17*). Several scaled-down chromatographical techniques were used. A summary including the most interesting comparative points is given in Table 3. Extracts obtained by the sonication procedure were suitable for purification, attaining, along with affinity chromatography, the highest factor of purification, although this technique requires a higher initial amount of sample and more time than the two FPLC-techniques.

Table 3. Comparison of different chromatographical techniques employed for lactase purification

	Gel filtration	Ion-exchange	Affinity
Factor purification	1.8	1.6	2.5
Yield/%	50	33	5.3
Initial amount of sample (mg)	2.4	2.4	12
Factor of dilution	1.5	1.5	1
Duration of the process/h	1	0.5	8
Reproductibility	+++	+++	++

Discussion

A key factor in the production and purification of intracellular enzymes is the cell disruption process (*18*). Various methods can be used to solubilize intracellular enzymes depending on their location inside the cell, intended use, and stability. Methods such as homogenization (*19–21*), sonication (*22–24*) and chemical extraction (*8*,*9*) have been used to release intracellular components. The procedure by which cells are disrupted has an important effect on the yield and quality of the protein extract (*15*).

In this paper we have compared the isolation of β-galactosidase, an intracellular protein, from *K. lactis*, *(i)* with different solvents, *(ii)* mechanical (glass-beads, vigorous stirring and sonication) and *(iii)* enzymatic procedures. The sonication procedure yielded the best results, obtaining an average of 34-fold more protein and 6600-fold more activity than in the other two methods.

The maximal percentage of released activity with the solvent extraction procedure was obtained with methanol after 21 h of contact with the extraction buffer. However, the absolute values of β-galactosidase activity were very low and therefore this procedure is not considered useful as a first step in the isolation and purification of intracellular materials.

The enzymatic extraction procedure introduces a new additive to the extract that may produce a downstream purification problem, in addition to the price of

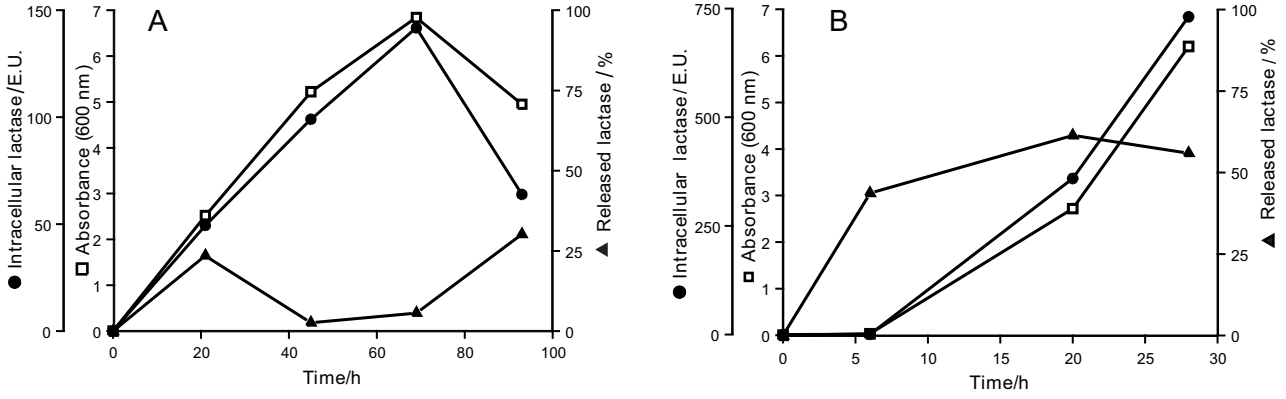

Fig. 3. Percentage of lactase released after the enzymatic treatment; cells grown in YPD (A) and YPL (B) were treated with lyticase. Data are given as percentage of released activity in the medium in reference to intracellular activity (in all cases SE<10 %)

the enzyme and the loss of β-galactosidase activity detected during the incubation.

Sonication of *K. lactis* cells proved to be the most effective method for this initial phase in the preparation of lysates for later purification. The crude protein extract obtained by sonication was fitted to support a purification process by means of chromatographical techniques.

The physiological state of the cells and the conditions of growth at the time of extraction have a major effect on disruption kinetics. Cells harvested during the log phase of growth are more easily disrupted than those harvested during the stationary phase. The best percentages of released activity obtained both in the sonication procedure and in the enzymatic extraction procedure were in the log phase of growth. Cells grown at a higher specific growth rate are probably easier to disrupt than cells grown at a lower specific growth rate, since they direct the available energy towards reproduction rather than towards synthesis or strengthening the wall structure (*1*). This would explain why *K. lactis* cells with a fast growth in a lactose medium presented a higher percentage of released activity after the enzymatic extraction procedure than cells growing slowly in a medium with dextrose.

Conclusions

The sonication extraction procedure proved to be the most effective technique for the recovery of β-galactosidase from *K. lactis* cells. The physiological state of the cells as well as the growth conditions at the time of disruption are a critical parameter for obtaining a good efficiency. The protein extract proved to be suitable for further chromatographic analysis. The application of this technique to other enzymes is of interest.

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Ekstrakcija intracelularnih proteina iz *Kluyveromyces lactis*

Sadržaj

Uspoređeno je nekoliko postupaka dobivanja sirovih ekstrakata iz kvasaca kako bi se postiglo dobro iskorištenje na intracelularnim proteinima. Tako se dobiva uzorak dobre kakvoće pogodan za daljnju kromatografsku analizu kao što je pokazano na primjeru β-galaktozidaze ili laktaze. Očito je da je sonifikacija najuspješniji postupak, a za postizanje dobrog iskorištenja bitni su pokazatelji: fiziološko stanje i uvjeti rasta stanica u trenutku ekstrakcije. Opisani je postupak jednostavan, brz i primjenjiv za druge enzime koje se želi izdvojiti.