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METHOD OF OBTAINING A TECHNICAL PREPARATION OF β--GALACTOSIDASE FROM *KLUYVEROMYCES LACTIS* YEAST NRRL-Y-1118

The active preparation of β-galactosidase was obtained from yeast *Kluyveromyces lactis* NRRL-Y-1118 cultivated on ultrafiltrates of whey. Extraction, two-fold precipitation with acetone and ultrafiltration leads to a fivefold purification of the enzyme its activity being regained in 65%. This enzyme preparation containing whey proteins had a marked stability at 4°C.

In the past ten years there has been a great interest in β-galactosidase and its utilization in the hydrolysis of lactose in dairy products [7, 2].

Isolation and purification of this enzyme from many strains of bacteria, yeasts and moulds has become the subject of many studies. The highest efficiency has been achieved from the two following kinds of yeast: *Kluyveromyces lactis* and *Kluyveromyces fragilis* [9, 19]. Analytical techniques such as precipitation with $(NH_4)_2SO_4$ or acetone, molecular filtration, ion-exchange, chromatography of affinity were used to obtain a highly purified form of β-galactosidase from strains belonging to those two species of yeast. The properties of this β-galactosidase were characterized [21, 10, 1]. Works on obtaining technical preparations of this enzyme in world literature have almost exlusively the character of patents. In Poland in spite of the comprehensive works of Dłużewski et al. [4] and Poznański et al. [16] the production process of this enzyme has not introduced into practice. In the works mentioned above *Kluyveromyces fragilis* yeast were the source of β-galactosidase. The author of this paper showed in his earlier findings that from 10 strains of yeast described in literature as strains which most actively hydrolyze lactose, the highest efficiency of enzyme was obtained from *Kluyveromyces lactis* NRRL-Y-1118 [6]. The present paper is an attempt at working out a simple method of obtaining from these yeasts an active preparation of β-galactosidase in order to use it in the dairy industry.

MATERIALS AND METHODS

Lyophilizated yeasts *Kluyveromyces lactis* NRRL-Y-1118 were used as the initial material in the obtainment of the enzyme preparations. The mother culture came from Northern Regional Research Laboratory, Peoria I11 and wes kept during the experiments in liquid medium YM (Difco Laboratories Inc.) at 4°C.

The growth medium for yeasts was prepared from dried whey produced by New York Res. and Development Corp. After the reconstitution at 45°C to a concentration corresponding to 25% lactose, the whey was ultrafiltrated with the use of Abcor Model 225.

The growth medium obtained from ultrafiltrated effluents was diluted with water to the content of 15% lactose and enriched with 0.5%/$NH_4/_2SO_4$ and 0.5% K_2HPO_4. Next it was pasteurized at 95°C for 30 min. The culture was cultivated in New Brunschwick-made fermentor with aeration of 2 air volume on 1 volume of culture per 1 minute and with 4.5 pH at 28°C. Biomass was centrifugated after 24 hours with 3.000×g and was subjected to freeze-drying.

CHEMICAL REAGENTS

In the process of isolation 0.02M potassium-phosphate buffer at pH 7.0 was used. Buffer was prepared from 2.72 g anhydrous KH_2PO_4 dissolved in 800 ml water. After setting pH at 7.0 with 50% KOH, 20 mg $MnCl_2$, 4 H_2O (10^{-4}M) and 0.01M β-2-mercaptoethanol were added and supplemented with water to 1000 ml.

The origin of foreign reagents used in the work and the degree of their purity are given in the detailed description of the methods used. All the other reagents had an analytic degree of purity.

DETERMINATION OF THE ACTIVITY OF β-GALACTOSIDASE

The enzymatic activity was determined calorimetrically using substrate ONPG (orthonitrophenyl-β-D-galactopyranosid) produced by Serva, in compliance with the method recommended by the producer for the Maxilact preparation [17].

The quantity of μM ONP released from ONPG per 1 min. was taken as the unit of enzymaticactivity marked in this work as J.A.G. The specific activity of β-galactosidase was expressed as the quantity of μM ONP released by 1 mg enzyme protein.

DETERMINATION OF PROTEIN CONTENT

Protein was determined after Lowry's method with the Folina reagent [8]. Protein solutions for determination were prepared in potassium-phosphate buffer in 0.02 M at pH 7.0.

MOLECULAR FILTRATION ON SEPHADEX G-200

For this purpose a column sized 75×2.5 cm with sephadex G-200 in 0.02 M potassium-phosphate buffer with an addition of 10^{-4} M $MnCl_2$ and 0.01 M 2-mercaptoethanol was used. 20 mg enzymatic preparation was drifted. The eluate was taken in 10 ml fraction at a rate of outflow 10 ml per hour. Protein content in the eluate was observed continuously by measurements of absorption at the length of 280 nm using absorption meter LKB.

DISK ELEKTROPHORESIS ON POLYACRYLAMIDE GEL

Disk elektrophoresis was carried out according to the method described by Davis and Orienstein as applied to the apparatus produced by Quickfit [12]. Protein samples in an amount of 50-200 μg on one gel were drifted in 20% saccharose solution. Gels were coloured in 1% solution of amide black 10B in 7% acetic acid for 30 minutes, then they were discoloured by electro-diffusion and stored in 3% acetic acid. The gels for the activity of β-galactosidase were coloured according to the method of Erikson and Steers [5] by incubation of 0.025% BNG (6-bromo-2-naphtylo-β-D-galactopiranosid) in 10% methanol v/v in 0.02 M potassium-phosphate buffer at pH = 7.0. After 10 minutes incubation gels were transferred to diazotizated blue B (1 mg/ml). Pink strips on gels showed the presence of an active form of β-galactosidase.

Reagents BNG and diazotizated blue B came from Sigma Chemicals LTD.

DETERMINATION OF PROTEOLYTIC ACTIVITY

The proteolytic activity in the enzymatic preparation of β-galactosidase was determined versus the specific substrate made of leather powder (Galbiochem) with remazol blue "Rimazolbrillant" (Farbwerke Hochest AG. Frankfurt/M) according to a method of Rinderknecht et al [5] and versus casein according to a method of Anson and Kunitz as modified by Nomoto and Narahashi [13].

RESULTS AND DISCUSSION

1. ISOLATION AND PRELIMINARY PURIFICATION

In order to get a technical preparation of β-galactosidase with a high activity all the methods applied in the isolation procedure presented below could be used on a technical scale.

In order to release the enzyme, yeasts cells in the form of 10% suspension were subjected to the process of disintegration in a homogenizer DYNO-MILL KDL produced by Bachofen AG-Schweiz whose efficiency parameters reach 200 l/h. According to data from literature the use of disintegration of that type guarantees a 90-100% extraction of soluble proteins [11]. A detailed scheme of the isolation process is shown in Fig. 1.

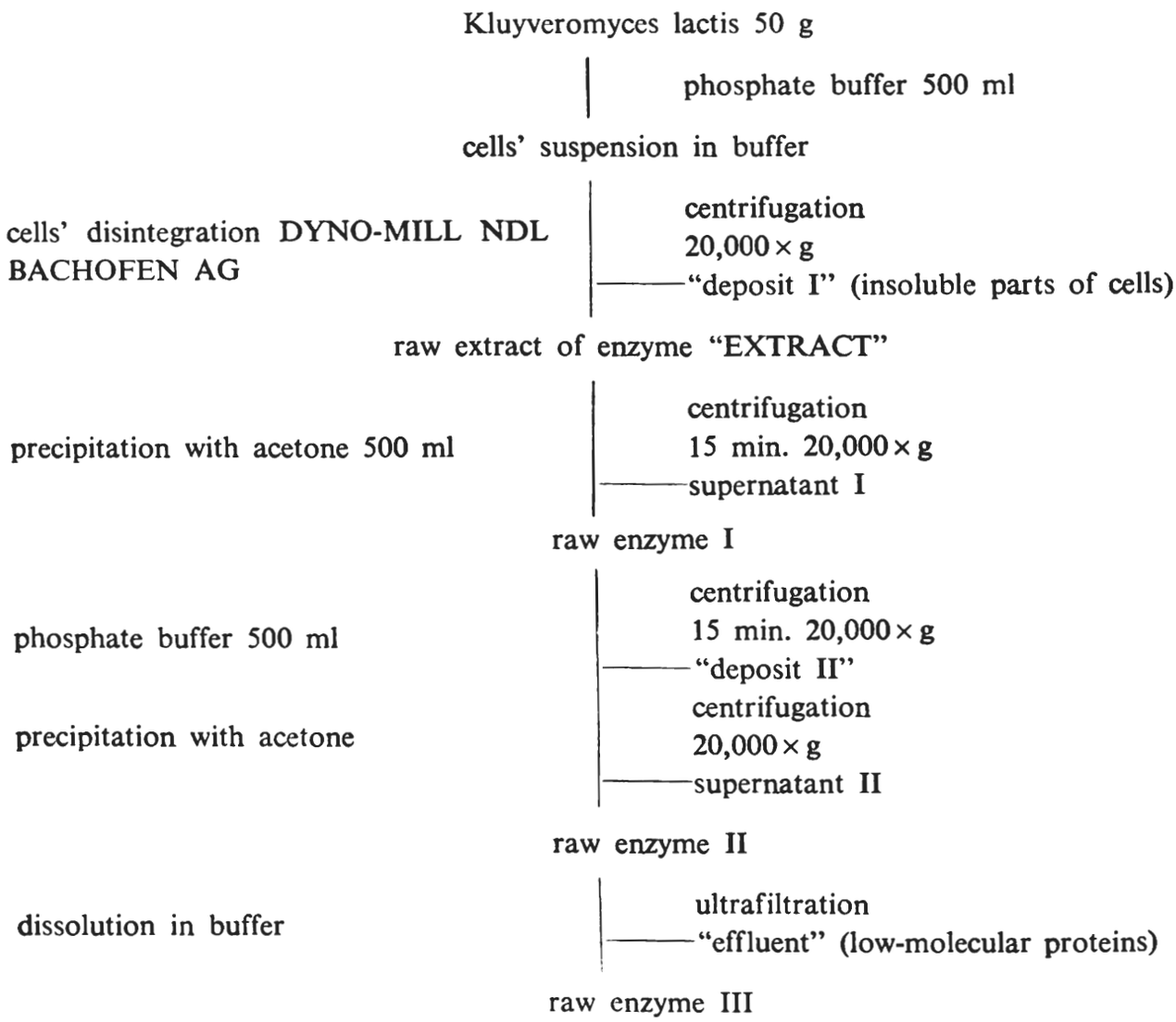

Fig. 1. Scheme of isolation process of β-galactosidase from yeast *Kluyveromyces lactis* NRRL-Y-1118

As shown on Fig. 1 the extract was separated from the suspension of divided cells by rotation (15 min. 20.000×g) and next the enzyme was isolated from it by a two-fold precipitation with acetone. Suspension of yeast cells and the consecutive processes of deposit dissolution were done in 0.2 M potassium-phosphate buffer. The enzymes deposit after two-fold

Table 1. Isolation and initial purification of β-galactosidase from yeast *Kluyveromyces lactis* NRRL-Y-1118

	Efficiency of β-galactosidase preparation from 50 g yeast in g dry matter	Content of protein in mg/g d.m. of preparation	Activity of β-galactosidase in J.A.G./mg protein	Specific activity of β-galactosidase in J.A.G./mg protein	Total activity of β-galactosidase J.A.G. 10^{-3}	Degree of enzyme purification	The enzyme efficiency in relation to extract in %
Raw extract	15.25	465	12.3	26.45	187.57	1	100
Acetone deposit I	3.13	580	49.80	85.86	156.00	3.24	83.17
Acetone deposit II	2.25	650	61.97	95.33	139.44	3.60	78.09
Raw enzyme (ultrafiltration)	1.40	690	87.15	126.52	122.35	4.78	65.23
Cells deposit after extraction	37.65		0.93		35.38		

precipitation with acetone was dissolved and ultrafiltrated by means of AMICON TCF 10 using films type XM100A. Ultrafiltration was carried up to a ten-fold density level. During the process buffer was added twice. Temperature was 4°C. The results of isolation and preliminary purification are given in Table 1.

During the experiment 140 mg lyophilizated preparation of β-galactosidase preparation of β-galactosidase with high activity (87,000 J.A, G/g) was obtained from 50 g of yeast (3% water).

This activity is twice higher than that guaranteed by the firm for the fresh preparation Maxilact^{R}-40.000. In the process of purification the specific activity of the enzyme grows almost five times with a simultaneous recovery of β-galactosidase (65% of the quantity contained in the extract). A two-fold precipitation with acetone causes a three-and-a-half times purification of the enzyme from nucleic acids and nucleoproteins. This is shown by the increase of the indicator A_{280}/A_{260} from 0.42 for raw substrate to 1.39 for "acetone deposit II" dissolved in phosphate buffer. Ultrafiltration raises the degree of purification to ca 5 by eliminating from the preparation of proteins with molecular weights lower than 100.000 D.

The above observations are aditionally confirmed by a comparison of the diagrams of molecular filtration of raw extract and enzymes preparation on Sephadex gel G-200, Fig. 2 and 3. The scheme of isolation and purification of enzyme shown on Fig. 1 leads to an elimination from the preparation of considerable amount of proteins with molecular weights higher and lower than β-galactosidase. However a preparation obtained in this way remains heterogeneous and has two protein peaks. The complex

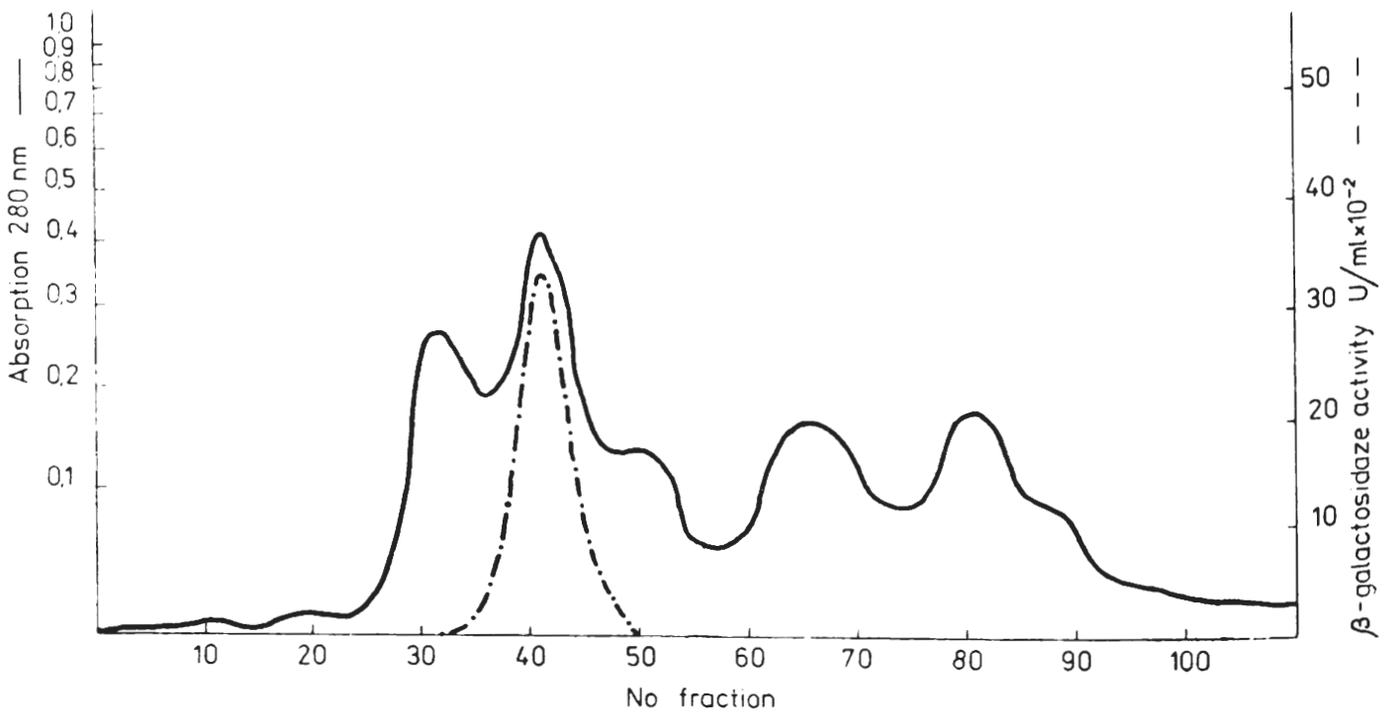

Fig. 2. Column chromatography of cells extract on Sephadex G-200. Column 75×2.5 cm. Samples (200 mg preparation) were eluated with phosphate buffer KH_2PO_4 — 0.02 M; pH = 7.0. Rate of outflow 10 ml/hour. Fractions 10 ml

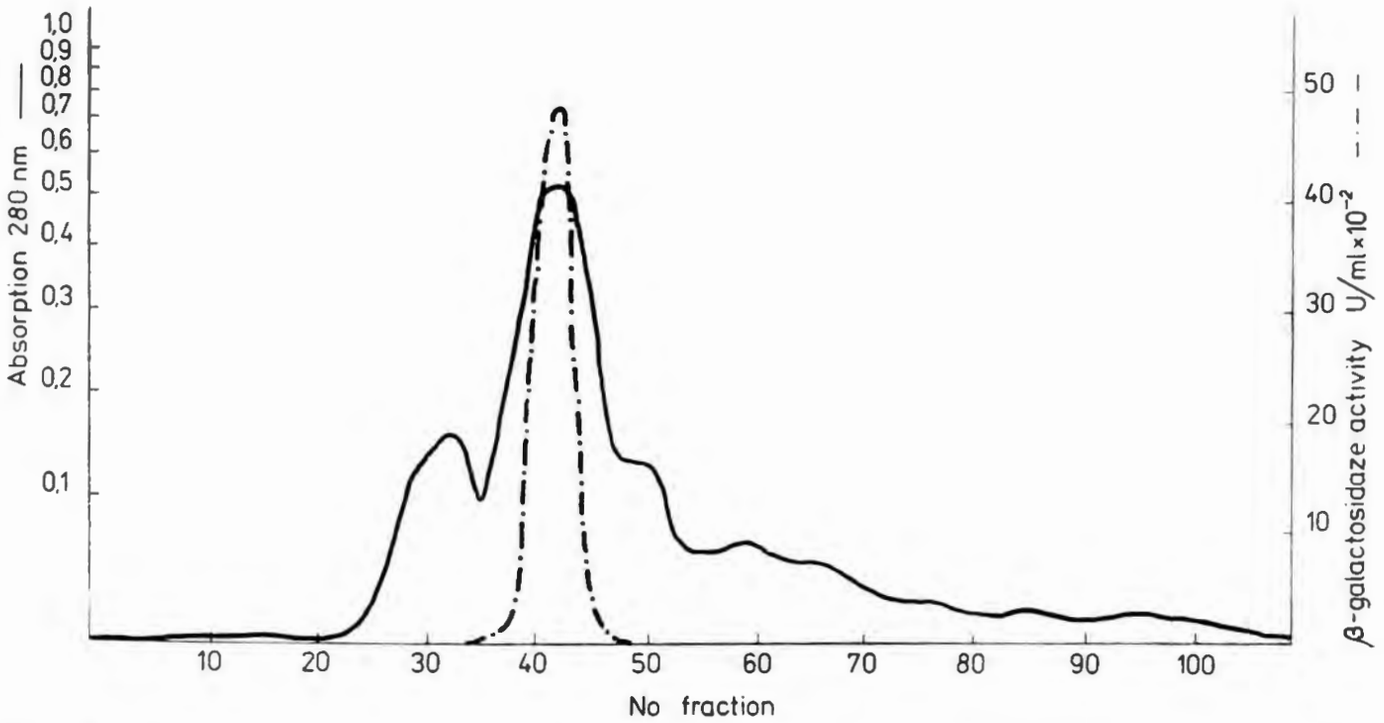

Fig. 3. Column chromatography "raw enzyme" on Sephadex G-200. Column 75×2.5 cm. 200 mg of preparation eluated with 0.02 M potassium-phosphate buffer; pH 7.0+10^{-4} M $MnCl_2$+0.1 M 2-mercaptoethanol. Rate of outflow 10ml/hour. Fractions 10 ml.

protein composition of "raw enzyme" is seen still better in conditions of electrophoresis on polyacrylamide gels presented on Fig. 4. In these conditions the comparatively analysed preparation Maxilact also proved

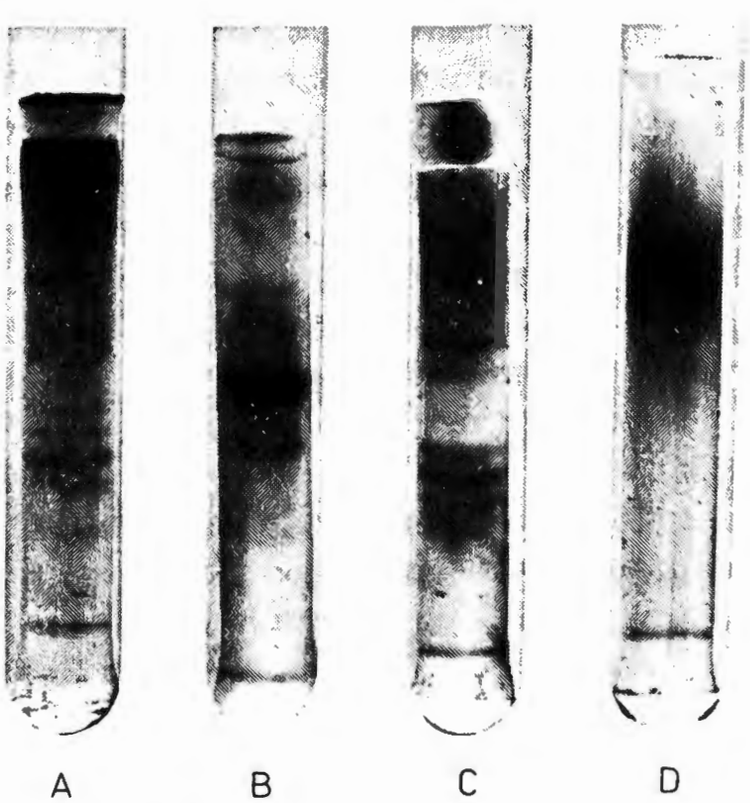

Fig. 4. Disk electrophoresis on polyacrylamide gel; A — cell extract, B — "raw enzyme", C — Maxilact preparation, D — "raw enzyme" coloured to find the activity of β-galactosidase with the use of BNG and diazotizated blue B. Gels A, B, C coloured with 1% amide black

to be heterogeneous, in spite of the fact that on sephadex G-200 it did not undergo disruption giving only one protein peak with the same range of activity as β-galactosidase. In spite of being heterogeneous, the "raw enzyme" obtained in this work did not show any proteolytic activity neither in relation to casein nor to specific substrate of leather powder with rimazol blue. The way of carrying out determinations is given in the description of methods. The analysis of results presented in Table 1 indicates that after extraction a large amount of the enzyme — about 18% of total activity remains among the divided cells. This could not be changed either by the use of a two-fold and three-fold process of cells desintegration or by an increase of ionic strength of the buffer used for the extraction. It seems that the rest of enzyme may well represent a form which cannot be released by mechanical methods and is linked with the structure of cells.

2. THE CHOICE OF FILLERS FOR THE TECHNICAL PREPARATION OF β-GALACTOSIDASE

In the technology of enzymatic preparations purified enzymes are usually mixed with fillers in order to increase their durability during storage and to make it easier to use them in technological conditions. The fillers used are mostly constituted by inorganic inert compounds, substrates and products of enzymatic reaction or those which have protective qualities of the protein albumin type.

In this work, the materials selected for the experiments were lactose, glucose and whey proteins obtained by the author by the method of whey ultrafiltration. The "raw enzyme" was mixed with fillers in three proportions 1:4, 1:3, 1:2. Enzymatic preparations obtained in this way had a water content below 2% water. The activity of β-galactosidase was determined in them just after they were obtained and after 6, 12, 18 and 24 months of storing them at 4°C. The results are given in Table 2.

In accordance with data presented in Table 2 the fillers used in the experiment had no influence on the activity of the enzyme contained in the preparations. The initial activity in those preparations is lower then the activity of the "raw enzyme" proportionally to the quantity of filler added. Besides, the preparations have good functional qualities (friability, solubility) and activity which can be regulated by the quantity of filler added.

The higher decrease of activity of β-galactosidase during storage was observed in the purified preparation called in this work "raw enzyme". After one year storage at 4°C the decrease was 39.3% and after two years it was 66.8%. The process of decreasing the activity of β-galactosidase with fillers was smaller when the concentration of enzyme in the preparation was lower. The preparation containing whey proteins proved to be

Table 2. Influence of the time of storage on the activity of β-galactosidase in preparations with different fillers

"Raw enzyme" with filler (months)		glucose			lactose			whey proteins after ultrafiltration			"Raw enzyme" without filler
		1:4	1:3	1:2	1:4	1:3	1:2	1:4	1:3	1:2	
Initial activity of the preparation J.A.G./mg		17.20	21.60	29.0	16.85	21.30	29.10	17.0	22.15	29.27	87.15
Drop of activity of β-galactosidase in % of initial activity after storage (months)	6	5.13	6.26	6.53	3.58	4.85	5.21	0.00	0.00	0.00	35.18
	12	8.73	8.12	9.81	11.37	10.17	12.38	2.65	3.89	5.16	39.27
	18	17.34	19.32	18.47	21.16	23.16	23.87	11.50	13.96	13.78	47.05
	24	30.58	32.41	35.56	28.75	30.28	34.85	16.25	17.94	19.20	66.83

especially durable. These results seem to comply with the statements of Dahlquist et al. [3] about the protective characteristics of whey proteins for the activity of β-galactosidase.

Current trends in the food processing industry rely on the use of that enzyme in continuous processes in a form bound on carriers [14, 20] or in ultrafiltration reactors [18].

The use of yeast β-galactosidase in an immobilizated form is still limited by the lack of its stability in the suggested preparations. Therefore taking into account the present state of knowledge and practice in the field of industrial utilization of β-galactosidase it can be said that a preparation obtained according to the described procedure may be used in the dairy industry especially as, thanks to its activity at 4°C, it is suitable for hydrolysis of lactose while storing milk in warehouse containers.

CONCLUSIONS

1. Extraction, two-fold precipitation with acetone and ultrafiltration of β-galactosidase after mechanical destruction of cells *Kluyveromyces* lactis NRRL-Y-1118 enables a five-fold purification of the enzyme and the obtainment of a preparation with high activity 87,000 J.A.G. on 1 g of preparation.

2. This enzyme has a complex protein composition both in conditions of molecular filtration (three peaks) and elektrophoresis on polyacrylamides (7 strips).

3. The utilization of whey proteins as a filler in enzymatic preparations of β-galactosidase (4 : 1) substantially prolongs its durability. Such preparation stored for 2 years at 4°C lost only 20% of its activity whereas the enzyme without filler lost in these conditions 66.8%.

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UZYSKANIE PREPARATU TECHNICZNEGO β-GALAKTOZYDAZY Z DROŻDŻY *KLUYVEROMYCES LACTIS* NRRL-Y-1118

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Streszczenie

Drożdże *Kluyveromyces lactis* hodowane na serwatce po ultrafiltracji z podwyższoną do 15% zawartością laktozy użyto jako źródło enzymu. Po dezintegracji komórek w homogenizatorze DYNO-MILL KDL f-my Bachofen enzym izolowano z surowych ekstraktów przez dwukrotne strącanie acetonem i ultrafiltracją (rys. 1).

W wyniku takiego postępowania z 50 g drożdży uzyskano 1400 mg preparatu β-galaktozydazy o wysokiej aktywności 87 000 J.A.G. (jednostek aktywności β-galaktozydazy-μmoli ONP uwolnionych z ONPG/min). W procesie oczyszczania nastąpił 5-krotny wzrost aktywności specyficznej enzymu przy jednoczesnym jego odzysku w granicach 65% (tabela 1).

Uzyskany "enzym surowy" charakteryzował się złożonym składem białkowym w warunkach sączenia molekularnego na sefadeksie G-200 (rys. 2 i 3) i elektroforezy na poliakrylamidach (rys. 4). Trwałość enzymu przechowywanego w formie zliofilizowanej wzrastała wyraźnie po zmieszaniu go w stosunku 1 : 4 z białkami serwatki (tabela 2). Aktywność β-galaktozydazy w takim preparacie wynosiła 17 000 J.A.G./g. W trakcie 2 lat przechowywania w 4°C utracił on jedynie ok. 16% aktywności (tabela 2).