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INFLUENCE OF DISINTEGRATION OF *SACCHAROMYCES UVARUM* YEAST CELLS OF FREEZE-DRYING OF ENZYMATIC EXTRACTS AND OF THE CONDITIONS OF STORAGE ON THEIR ENZYMATIC ACTIVITY

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Key words: brewery yeast, proteolytic activity, method of disintegration, of yeast cells, freeze drying, fermentation of sugars.

Changes of proteolytic, fermentation and decarboxylation activity during the process of freeze drying and storage of enzymatic extract obtained from yeast disintegrated by the mechanical-pressure method and with cooled acetone were investigated. It has been found out that lyophylization does not have any significant influence on the enzymatic activity of the extracts tested. The enzymes washed out from the sediment of acetone-cooled disintegrated cells were more stable during storage than extracts dried by freezing.

INTRODUCTION

The stabilization of protein substance, especially enzymes, is of essential significance. It is important both in the production of enzymes and in all the areas of their application and, above all, in identifying the particular properties of enzymes.

Enzymatic preparations in a hydrated state indicate a much higher stability than preparations in the form of concentrates or solutions. A traditional method of preserving proteins is their drying. Inactivation of enzyme solutions dried at room temperature is usually impossible to avoid. Drying in a high vacuum, at low temperature, in a frozen state is an efficient method of preserving enzyme activity [4].

In the present paper we present data concerning the influence of freeze-drying on the enzymatic activity of intracellular and cellular wall

extracts of *Saccharomyces uvarum* yeasts (*Saccharomyces carlsbergensis*) [7] and changes in enzymatic activity taking place during the storage of preparations.

ORGANISATION OF THE EXPERIMENT AND METHODS

1. Five strains of the *Saccharomyces uvarum* (*Saccharomyces carlsbergensis*) yeasts: ATCC, B_2, D_1, M_{15}, and SW selected from among many one investigated in our earlier tests [2, 3] were used in the experiment. Yeasts were incubated on wort-agar slant and next washed with several millilitres of sterile medium — non hopped first wort. Cultures were placed in New Brunswick FS-314 fermentors at 30°C, pH of the medium 4.6 and aeration 1 $m^3/h/l$ of the nutrient.

2. Method of obtaining enzymatic extracts. Yeasts cells were centrifugated from the medium during 15 min, at 1500 g. The cell sediment was diluted in sterile distilled water and centrifugated again. The biomass was disintegrated by two methods: with acetone cooled down to — 20°C acc. to a method described by Keil and Sormova [5] and mechanically using a disintegrator type Biotec — X25, pressure 200 MPa.

After disintegration, the cell biomass was divided into two fractions — intracellular (soluble in phosphate-citrate buffer, pH 6.6) and of the cellular wall (soluble in 2M NaCl solution) acc. to methods described by Westhoff et al. [13] and Mendoza et al. [9].

A detailed description of the method has been presented in our earlier works [2, 3].

Prior to lyophilization, NaCl was removed from cellular wall extracts by dialysis to distilled water using an Amicon DC-2 dialyser. After lyophilization, the samples were kept at 4°C for 60 days. Enzymatic extracts were reproduced by a dissolution of lyophilization in sterile distilled water to the volume of the sample prior to lyophilization.

3. Assessment of the enzymatic activity of extracts reproduced from lyophilizates directly after lyophilization and in extracts made from samples stored for 30 and 60 days:
— protein content by Lowry's method [8],
— proteolytic activity acc. to Westhoff et al. [13],
— fermentation activity of glucose and raffinose [3],
— decarboxylation activity of aminoacids acc, to the modified method of Soda et al. [12] using — as a substrate — a water solution containing 0.125% of each of the following aminoacids: DL-valine, DL-trypsin, DL-glutamic acid, DL-phenylalanine, DL-histidine, DL-arginine and DL-tyrosine. The results were expressed in $\mu L\ CO_2/1$ mg protein of the enzymatic extract examined.

DISCUSSION

Neither the results nor the statistical assessment have shown that lyophilization has a significant effect on the change of solubility of protein comprized in intracellular and cellular wall extracts or on their enzymatic activity (Table 1 A). Small differences in protein content and the proteolytic, fermentation and decarboxylation activity od enzymatic extracts before and after lyophilization (Table 1 A) resulted rather from an error of the determination methods than from changes produced by freeze-drying.

On the other hand, significant changes were observed in enzymatic activity and in protein content in extracts after 30 days storage at 4°C (F calculated $>$, $F_o$read F distribution tables at a confidence interval $\alpha = 0.01$). The average amount of protein in intracellular extracts was by 5.47% and in cellular wall extracts by 5.96% lower (Table 1 B) than in corresponding extracts obtained directly after lyophilization (Table 1 A). This shows that the solubility of enzymatic protein comprized in the structures tested dropped during storage. Proteolytic activity dropped by an average of 1.88% only. But the fermentation activity of sugars was lower by 65-70% and cellular wall extracts proved somewhat more stable than intracellular ones. Also the decarboxylation activity of aminoacids by the enzymatic extracts tested after 30 days storage was lower by an average of 32.5% than the decarboxylation activity of extracts obtained directly after lyophilization.

A distinct decrease of the solubility of enzymatic protein took place during the next 30 days of storage of the freeze-dried extracts: in intracellular extracts by an average of 32.4% and cellular wall extracts by 23.8% (Table 1 B) compared with extracts directly after lyophilization. The same trend was observed in the case of proteolytic activity (decrease by 38.9%). The decrease of fermentation activity of the extracts tested after 60 days of storage amounted to an average 9.2% in intercellular extracts and 16.8% in cellular wall extracts compared with the samples after 30 days storage. However, the activity observed constituted only 16.9% of the initial fermentation ability of glucose and 21.4% of the initial fermentation ability of raffinose. After 60 days storage at 4°C, only 51% of the initial decarboxylation activity of aminoacids was preserved by the enzymatic extracts tested.

Enzymatic extracts preserved by means of freeze-drying displayed the lowest stability during storage at 4°C in relation to the activity of sugar fermentation and aminoacids decarboxylating enzymes. The changes in protein content observed in enzymatic extracts during storage were probably due to denaturation changes which led to a reduced solubility of protein.

Table 1A. Enzymatic activity of intracellular (W) and cellular wall (S) extracts from yeasts disintegrated by the mechanical-pressure method

Strain	Type of extract	Enzymatic extracts prior to freeze-drying					Lyophilized enzymatic extracts				
		protein content (mg/ml)	proteolytic activity (J.A./1 mg protein	fermentation activity		decarboxylation activity (μlCO_2/1 mg protein	protein content mg/ml	proteolytic activity (J.A./1 mg protein	fermentation activity μlCO_2/1 mg protein		decarboxylation activity (μlCO_2/1 mg protein
				glucose	raffinose				glucose	raffinose	
ATCC	W	10.98	104.90	150.90	150.62	9.61	11.03	110.16	153.00	148.19	10.03
	Ś	6.13	180.61	234.35	231.10	15.31	7.14	176.83	228.40	229.07	14.90
B_2	W	4.82	208.17	173.14	208.31	12.13	5.04	202.75	180.71	211.44	10.83
	Ś	3.11	248.93	193.53	217.53	17.21	2.98	282.73	189.75	223.82	18.08
D_1	W	9.51	245.42	153.80	197.48	13.14	8.95	239.12	147.62	204.91	12.95
	Ś	4.09	305.31	188.60	158.59	12.70	3.97	299.83	191.43	156.00	13.15
M_{15}	W	12.65	229.30	163.60	149.20	27.67	12.91	233.19	159.57	150.17	29.00
	Ś	5.21	209.17	208.75	198.65	20.29	6.01	210.00	211.00	192.33	22.13
SW	W	4.89	145.75	174.50	161.53	19.00	5.07	151.72	180.73	170.95	20.07
	Ś	3.13	239.75	203.57	206.00	18.09	3.40	233.87	201.41	195.38	16.95

Table 1B. Changes in enzymatic activity during the storage of lyopholized intracellular (W) and cellular wall (S) extracts from yeasts disintegrated by the mechanical-pressure method

Strain	Type of extract	Lyophilized enzymatic extracts stored at 4°C for 30 days					Lyophilized enzymatic extracts stored at 4°C for 60 days				
		protein content mg/ml	proteolytic activity J.A./1 mg protein	fermentation activity μl CO_2/1 mg protein		decarboxylation activity μl CO_2/ /mg protein	protein content mg/ml	proteolytic activity J.A./1 mg protein	fermentation activity μl CO_2/1 mg protein		decarboxylation activity μl CO_2/1 mg protein
				glucose	raffinose				glucose	raffinose	
ATCC	W	10.91	99.60	50.48	46.67	6.63	7.52	97.11	20.16	25.00	2.99
	Ś	6.89	160.00	81.50	71.09	7.19	4.72	120.89	17.47	36.74	6.55
B_2	W	4.65	207.30	46.24	62.96	9.71	3.80	199.75	35.11	49.87	8.65
	Ś	3.07	240.17	69.77	70.85	12.14	2.56	109.25	56.03	45.71	9.50
D_1	W	8.83	241.15	73.39	54.85	10.90	6.68	113.83	42.40	47.92	8.90
	Ś	3.75	305.48	91.66	84.79	12.22	3.60	160.71	25.87	42.91	7.55
M_{15}	W	11.30	218.73	55.51	36.81	11.18	7.12	98.64	19.65	33.36	11.19
	Ś	5.20	216.65	92.51	42.95	18.29	4.16	130.59	12.31	35.01	5.44
SW	W	4.98	150.59	26.99	56.61	15.68	3.91	90.13	35.91	46.28	5.62
	Ś	3.17	229.75	28.16	61.48	9.50	2.88	160.57	44.23	37.62	7.47

After 30 days storage no significant changes were observed in the content of protein and the enzymatic activity of extracts obtained from the stored sediment of cells disintegrated with cooled acetone (Table 2B).

Substantial modifications in protein solubility and enzymatic activity of the extracts obtained were observed after 60 days storage of the sediment of cells disintegrated with acetone. Both the intracellular extract and the cellular wall extracts showed a lower content of protein (29.7 and 23.7% respectively, Table 2B), than the content of protein in extracts obtained from the sediment of disintegrated cells prior to storage (Table 2A).

Table 2A. Enzymatic activity of intracellular (W) and cellular wall (S) extracts from yeasts disintegrated with cooled acetone

Strain	Type of extract	Protein content	Proteolytic activity J.A./1 mg protein	Fermentation activity µl CO_2/1 mg protein		Decarboxylation activity µl CO_2/1 mg protein
				glucose	raffnosie	
ATCC	W	14.40	100.17	115.53	132.28	6.63
	Ś	7.20	150.00	62.89	92.27	6.19
	W	12.56	198.50	89.54	126.00	10.65
B_2	Ś	3.22	75.75	111.77	127.18	19.50
	W	9.60	130.95	75.02	124.44	13.90
D_1	Ś	5.80	312.19	156.88	177.31	11.55
	W	7.80	115.00	139.39	112.03	22.20
M_{15}	Ś	6.40	140.00	186.89	130.09	23.29
	W	7.02	149.98	103.18	121.79	15.68
SW	Ś	2.78	82.30	123.03	162.45	17.47

It should be assumed that the solubility of protein comprized in the sediment of disintegrated yeast cells was decreased during storage. Proteolytic activity also decreased by an average of 35.5% in the case of intracellular extracts and by 12.3% in cellular wall extracts as compared with the proteolytic activity of extracts obtained from the sediment of cells directly before disintegration. Enzymes fermenting sugars featured a higher stability during storage in the case of intracellular extracts (average drop by 15.98%) than in extracts of the cellular wall (drop by 28.6%). But the decarboxylation activity of aminoacids decreased after 60 days storage by an average of 40.48%, and in cellular wall extracts by 27.95% only.

Enzymes washed out from the stored sediment of cells disintegrated with cooled acetone were more stable during storage than enzymatic extracts obtained from the cells of yeasts disintegrated by the mechanical-pressure method, and preserved by freeze-drying. Most probably, the role of a protective agent was played by the structural proteins of yeasts cell.

Enzymatic extracts obtained from yeasts disintegrated by the mechanical-pressure method and preserved by freeze-drying displayed a very

Table 2B Changes in the enzymatic activity of intracellular (W) and cellular wall (S) extracts during storage of yeasts disintegrated with cooled acetone

Strain	Type of extract	Enzymatic extracts obtained from yeasts disintegrated with cooled acetone stored for 30 days at 4°C					Enzymatic extracts obtained from yeasts disintegrated with cooled acetone stored for 60 days at 4°C				
		protein content mg/ml	proteolytic activity J.A/1 mg protein	fermentation activity µl CO_2/1 mg protein		decarboxylation activity µl CO_2/ /mg protein	protein content mg/ml	proteolytic activity J.A/1 mg protein	fermentation activity µl CO_2/1 mg protein		decarboxylation activity µl CO_2/1 mg protein
				glucose	raffinose				glucose	raffinose	
ATCC	W	14 90	98.70	113.95	131.00	6.60	11.46	97.11	102.61	111.07	2.99
	Ś	7.04	150.00	63.03	90.93	6.12	4.02	120.89	59.07	81.63	3.65
B_2	W	12.60	200.04	90.17	125.70	10.35	5.10	149.75	88.63	100.29	9.14
	Ś	3.35	75.30	113.00	125.18	19.45	2.94	77.50	94.64	72.26	17.12
D_1	W	9.50	129.83	72.00	120.95	14.00	7.40	113.83	70.14	119.56	7.19
	Ś	5.80	307.20	153.88	169.37	11.03	2.78	160.71	125.36	109.29	7.12
M_{15}	W	7.75	113.80	138.94	112.80	21.80	5.94	98.64	84.64	110.98	15.28
	Ś	6.50	140.00	185.75	131.00	23.12	3.66	130.59	124.51	88.85	18.54
SW	W	6.99	150.10	102.13	119.31	14.75	6.18	150.13	95.82	72.91	6.50
	Ś	2.80	82.80	121.46	161.79	16.90	2.50	90.07	98.68	88.88	9.75

low stability during storage. Similar results have been obtained by Nowak [10] during storage of lyophilized proteases of the Saccharomyces cerevisiae yeasts. During freeze-drying various parts of a protein molecule form bonds with water. In this water strongly competes with hydrogen bonds in protein molecule. In addition, ions are released which may have an influence on the durability of ion bonds. However, these bonds have proved unstable during storage at 4°C. The choice of the protective agent is no problem in the technological process. On the other hand, in model tests, the addition of a stabilizer (polysaccharide or protein) makes it difficult to watch the process of proteolysis or sugar hydrolysis and fermentation and makes it impossible to quantitatively interpret the course of enzymatic reactions.

In this connection, it would be advisable to conduct research on the choice of a potective agent (stabilizer) in order to prolongate the durability of enzymatic preparations or to check other preservation methods e.g. spray drying, the method patented by Kornacki et al. [6].

CONCLUSIONS

1. Disintegration with cooled acetone as well as the mechanical pressure method have proved useful for the recovery of proteases and enzymes fermenting sugars from the cells of *Saccharomyces carlsbergensis* yeasts.
2. Intracellular and cellular wall extracts differed considerably in terms of enzymatic activity. Similar differences in enzymatic activity were observed for corresponding extracts obtained from various strains of yeasts.
3. Enzymes stored in the form of a sediment after disintegration with cooled acetone were more stable than enzymatic extracts preserved by freeze-drying.
4. In order to increase the stability of enzymatic extracts tested it is necessary to select and to use a protective agent.

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WPŁYW METODY DEZINTEGRACJI KOMÓREK DROŻDŻY *SACCHAROMYCES CARLSBERGENSIS*, SUSZENIA SUBLIMACYJNEGO EKSTRAKTÓW ENZYMATYCZNYCH ORAZ WARUNKÓW PRZECHOWYWANIA NA ICH AKTYWNOŚĆ ENZYMATYCZNĄ

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Streszczenie

Przebadano zmiany aktywności proteolitycznej, fermentacyjnej i dekarboksylacyjnej podczas procesu suszenia sublimacyjnego i przechowywania ekstraktów enzymatycznych z drożdży dezintegrowanych metodą mechaniczno-ciśnieniową oraz schłodzonym acetonem. Nie zaobserwowano by proces liofilizacji wpływał w istotnym stopniu na rozpuszczalność białka, bądź jego aktywność enzymatyczną. Istotne natomiast były zmiany aktywności enzymatycznej i rozpuszczalności białka podczas przechowywania w temp. 4°C zarówno liofilizowanych ekstraktów enzymatycznych, jak i osadu komórek dezintegrowanych schłodzonym acetonem (tab. 1B, 2B).

Ekstrakty enzymatyczne utrwalone metodą suszenia sublimacyjnego po 60 dniach przechowywania w temp. 4°C wykazały średnio: 71,9% początkowej zawartości białka, 61,1% początkowej aktywności proteolitycznej, 19,2% początkowej aktywności fermentacyjnej i 51,1% początkowej aktywności dekarboksylacyjnej (tab. 1B). Natomiast ekstrakty enzymatyczne otrzymane z przechowywanego przez 60 dni osadu komórek dezintegrowanych schłodzonym acetonem wykazały średnio: 73,2% początkowej zawartości białka, 76,1% początkowej aktywności proteolitycznej, 77,7% początkowej aktywności fermentacyjnej i 65,8% początkowej aktywności dekarboksylacyjnej (tab. 2B).

Na podstawie powyższych danych stwierdzono, że enzymy przechowywane w postaci osadu komórek dezintegrowanych schłodzonym acetonem były stabilniejsze niż ekstrakty enzymatyczne utrwalone metodą suszenia sublimacyjnego.