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LACTASE IN IMMOBILIZED CELLS OF WATERMELON

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A cell suspension culture of Citrullus vulgaris Schrad cv. "Samara" was permeabilized by Tween 80 and immobilized by glutaraldehyde. The highest lactase activity was achieved at pH 4.3, the temperature optimum for cell suspension was at 50°C, while for the immobilized cells the optimum was at 58°C. The hydrolysis of substrate was linear for 3 h, reaching 60–67% conversion rate. The cells were characterized by high enzyme activity. The stability of the enzyme showed convenient physico-mechanical properties (physical protection from shear forces and easy separation of product from biocatalysts) in long-term storage.

Key words: lactase, cell permeabilization, cell immobilization, watermelon.

Biotechnological production can be an alternative to limited resources of natural bioactive compounds. Cell and tissue cultivation techniques were applied in agriculture in plant propagation at the beginning. It was recognized later that the multifunctional enzyme system of viable plant cells can be used for biosynthesis and biotransformation of substances both of natural and synthetic origin [1, 2]. The quality of human nutrition is, besides other nutrients, dependent on the quality, quantity, structure, and physicochemical properties of sugars in food. Biotransformation of sugars plays an important role in some biotechnological processes [3–5]. The determination of lactose and galactosidase activities plays an important role in many fields of basic and applied research [6, 7].

Lactose utilization in dairy products is limited due to its poor solubility, insufficient sweetness, and laxative effect when consumed in large quantities. Lactase hydrolyzes lactose into glucose and galactose and has recently evoked considerable interest because of its application in the food industry, nutrition, and medicine [3, 4, 8]. It improves the product sweetness, encourages milk consumption by people who suffer from lactose intolerance, and increases the product quality and process efficiency in the dairy industry. It further creates an opportunity to produce sweeteners from whey and whey permeate instead of discharging them, which is a serious environmental pollution problem. Hydrolyzed whey permeate may be effectively used as a substitute for corn syrup in soft drinks, fermented beverages, and confectionery products [3, 9].

Lactase can be used to hydrolyze lactose in milk, whey, and whey permeate in a number of ways. The choice of process technology depends upon the nature of the substrate, the characteristics of the enzyme, and the economics of production and marketing of the product. The lactase can be used as a soluble enzyme or immobilized enzyme. The soluble enzyme is normally used for batch processes while the immobilized form is applied in continuous operations. Immobilized lactase systems remain more economically feasible than free systems. Biotransformation of lactose in milk with immobilzed lactose may be performed continuously with reutilization of the enzyme, resulting in a significant cost reduction. The most commonly used method, polymerization with glutaraldehyde, does not preclude applicability of resulting products in foods [3, 5, 9].

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TABLE 1. Lactase Activity in Cell Suspension and in Immobilized Cells of Watermelon

Cells	Protein (mg/g dry mass)	Activity (nkat/g dry mass)	Specific activity (nkat/mg protein)
Suspension	26.8±0.96	6.1±0.28	0.23
Permeabilized	9.5±0.59	5.1±0.23	0.54
Immobilized	9.4±0.61	4.8±0.23	0.51

TABLE 2. Stability of Lactase in Immobilized Watermelon Cells on Storage

Conservancy	Original activity in suspension culture (%)				
	0 month	1 month	2 month	3 month	6 month
None	69	-	-	-	-
CLCTC (50 mg/l)	68	69	71	80	92
ATDNO (100 mg/l)	68	70	72	82	93
Chloramphenicol (50 mg/l)	66	71	73	83	95
Sodium azide (200 mg/l)	65	72	74	86	97
Frozen in 0.15 M NaCl	66	72	75	87	98
Dried cells	67	72	76	87	98

CLCTC, chlortetracycline hydrochloride; ATDNO, (1-methyldodecyl)-dimethylamine-4-oxide; original activity = enzyme activity (100%) in cell, suspension without immobilization.

To date, Aspergillus niger, Aspergillus oryzae, Penicillim notatum, and Kluyveromyces marxianus lactases have been successfully bound to a variety of solid supports and used to hydrolyze whey [3, 6, 11]. However, while searching for new producers it is necessary to find sources with different enzyme activities.

Lactase (β-D-galactoside galactohydrolase EC 3.2.1.23) and β-galactosidase catalyse the hydrolysis of the terminal β-galactose linkage of glycosides. The enzyme is widely distributed in various plant tissues [12, 13]; however, the precise role of this enzyme is not well understood. It has been suggested that this enzyme is involved in the degradation of plant cell-wall polysaccharides in relation to cell growth, fruit ripening, and seed and pollen germination [14, 15]. Although lactase is generally present also in plants, this source has not been used previously.

Enzymes of living or nonliving microorganisms and animal and plant cells have been bound within each other or to carrier materials [11, 16, 17]. Immobilization techniques have had a great impact on enzyme technology nowadays [18].
In this paper the enzymic hydrolysis of lactose by free as well as by glutaraldehyde immobilized watermelon cells and their storage stability were studied. An immobilization technique without any soluble carrier was used. The cell were immobilized by cross-linking. In cells immobilized in this way the studied enzyme had very high activity for a long time.

Microscopic investigation of the imobilized cells compared with cell suspension showed hardly any morphological changes. A little thinning of cell walls after permeabilization was observed. Moderate cytoplasm plasmolysis and aggregation of cells after immobilization was observed too.

According to the respiration rate and vital staining (fluorescein or 2,3,5-triphenyltetrazolium chloride) cells immobilized by glutaraldehyde were not viable. Also the glucose was utilized only by cell in suspension, not by immobilized cells (Fig. 1).

The permeabilization of the studied cells by Tween 80 led to the leakage or degradation of proteins while the enzyme activity showed a moderate decrease, thereby the specific activity increased. By glutaraldehyde crosslinking a moderate fall in the enzyme activity has been found (Table 1).

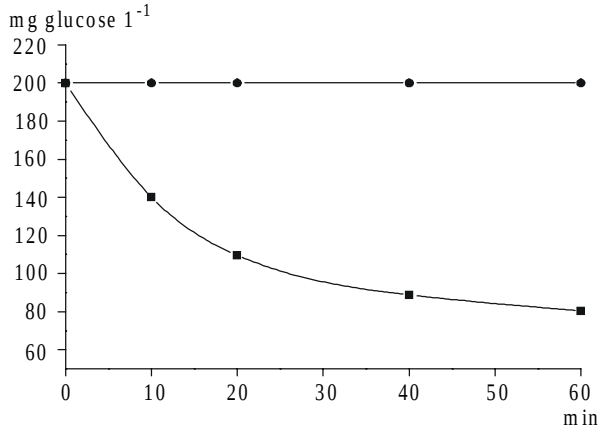

Fig. 1

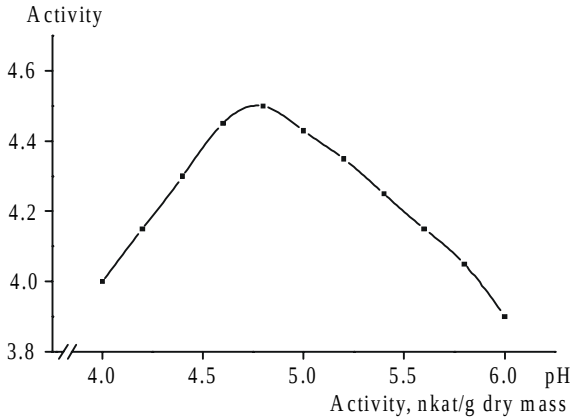

Fig. 2

Fig. 1. Time course of glucose utilization by cells immobilized with glutaraldehyde (circles) and by cells in suspension (squares).

Fig. 2. pH optimum of lactase in immobilized cells of watermelon.

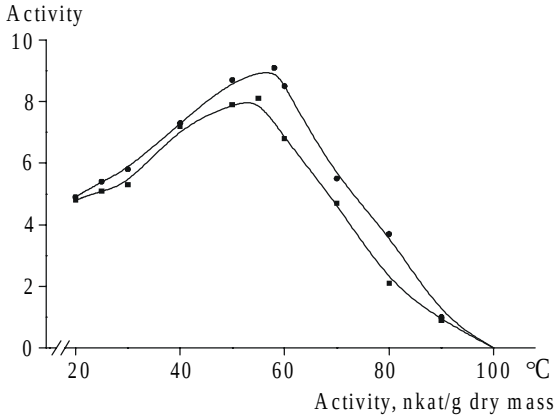

Fig. 3. Effect of temperature on activity of lactase in immobilized watermelon cells (circles) and in cell sussension (squares).

Saccharose is probably the most widely used carbon source in plant tissue cultures. After inversion of saccharose, glucose and fructose are present in the media in roughly equal amounts during the first few days, but the cells did not consume fructose until glucose is present. The cells immobilized in alginate gels utilized glucose while the glutaraldehyde crosslinked cells did not (Fig. 1) [19].

Lactase in immobilized cells of watermelon had a pH optimum of 4.8 like viable cells in suspension (Fig. 2). Enzyme hydrolysis of (*p*-nitrophenyl-β-D-galactopyranoside) was linear for 3 hours, reaching 60–67% of substrate conversion, than practically stopped. The temperature optimum of enzyme activity at immobilized cells was 58°C and at cells suspension 50°C, indicating the relatively high degree of temperature stability of this hydrolases (Fig. 3).

Partially purified enzyme preparations of lactase from gherkin and poppy seedlings were inhibited by galactose and glucose in a moderate way [12]. A similar inhibitory effect was observed in immobilized cells, too.

The inhibitory effect of 0.1–0.5 mM *p*-chlormercuribenzoic acid on lactase can be eliminated with and 5–10 mM cysteine, dithiothreitol, or 2-mercaptoethanol [2]. These results indicate that SH-groups are essential for the enzyme activiti es of both lactase and α-galactosidase [19].

As illustrated in Table 2, the activity of the enzyme in watermelon cells immobilized by glutaraldehyde (in 0.15 M NaCl with all preservatives tested) during 6 months' storage is still relatively high. The same phenomenon (an increase of α- and β-galactosidase during storage) was observed in immobilized cells of poppy and ginseng [1, 2, 9]. The observed increase in the

activity on storage remains unclear. It might be due to a gradual dissociation of inhibitory compounds originally interacting with the enzyme. The tested preservatives do not influence the enzyme activity.

The immobilization costs are very low, and no special equipment is needed. Aeration, agitation, and the kind of cultivation medium have no influence on the biotransformational potential of glutaraldehyde immobilized cells. Immobilization of the cells makes enzyme isolation unnecessary, whereas the specific enzyme activity of biocatalysts remains quite high [20]. Cells immobilized by glutaraldehyde (by crosslinking) compared with cells immobilized by entrapment in beads (alginate, carrageenan or other matrices) bring important advantages in the activity of the enzymes.

Many authors studied the structure of glycosidases and the effect of different effectors on their activity [21, 22]. Lactase and other glycosidases can be prospectively applied in biotransformation processes in the food industry and also in pharmaceutically important compounds; their application in structure studies of these compounds is another possible practical application [3, 4, 7, 23–25].

EXPERIMENTAL

Tissue Cultures. Long-term tissue cultures and cell suspension were derived from watermelon seedlings Citrullus vulgaris cv. "Samara" as was previously described [26].

Cell Permeabilization. Cell suspensions were filtered through a nylon cloth and 15 g of fresh mass and suspended in 50 ml of 5% of Tween-80 in 0.15 M NaCl solution. Permeabilization proceeded for 3 h under moderate stirring at 20°C. The cells were filtered off and washed first with 3 liters of distilled water and than with 2 liters of 0.15 M NaCl solution.

Immobilization. The permeabilized cells were immediately resuspended in 50 ml of 0.15 M NaCl solution, and 5 ml of 25% glutaraldehyde was slowly added under mild stirring at room temperature for 2 h. Immobilized cells were washed with 2 liters of distilled water and 2 liters of 0.15 M NaCl solution and separated by filtration.

Fresh and Dry Mass Determination. Fresh and dry mass of cell suspensions were determined gravimetrically. For the determination of dry mass, samples were dried to constant mass at 105°C.

Storage Stability. The stability of lactase during storage was monitored in the following experiments. The immobilized cells were stored at 4°C in 0.15 M NaCl supplied with following compounds: α-chloramphenicol 50 mg/l, β-chlortetracycline hydrochloride (CLCTC) 50 mg/l, c-(l-methyldodecyl)-dimethylamine-4-oxide (ATDNO) 100 mg/l [27]. These experiments were repeated at least three times.

Glucose Utilization. The immobilized cells and cell suspensions were exposed to an initial glucose concentration of 200 mg/l in the cultivation medium [28, 29] devoid of sucrose. The concentration of glucose was determined by the method of Trinder [30]. Cells immobilized by glutaraldehyde were dried for 24–36 h in a thin layer at laboratory temperature and were then stored in tightly closed polyethylene bottles at 4°C or 25°C, respectively. The dry biocatalyst needs to be soaked in water or buffer at least for 1–2 h prior to its application [26].

Enzyme Assay. The enzyme assay was performed by the modified method [31] using *p*-nitrophenyl-β-D-galactopyranoside (βPNG) as substrate. The reaction mixture contained 0.1 g of wet cells and 0.5 mg βPNG in 2 ml McIlvaine buffer, pH 4.8. The control contained boiled cells. Both mixtures were kept for 20 min to 5 h at 30°C on a rotary shaker (80 r.p.m.) and the reaction was stopped by addition of 2 ml of 1 M Na_2CO_3. Substrate conversion was calculated based on the decrease in substrate concentration following 3 h incubation. The nitrophenol released was determined spectrophotometrically at 420 nm. The cells were separated from the reaction mixture, dried, and the enzyme activity was calculated for 1 g of dry mass [18, 26].

The determination of enzyme activity was repeated at least five times and the enzyme activity is expressed in katals. Protein content was determined by the method of Bradford [32] using bovine serum albumin as a standard.

Cell Viability. This was determined by the method of [33] with 2,3,5-triphenyltetrazolium chloride (TTC) or fluorescein diacetate and oxygen electrode respectively.

REFERENCES

1. K. Weissova, J. Stano, K. Neubert, D. Kakoniova, P. Kovacs, K. Micieta, and D. Liskova, *Hort. Sci*., **28**, 145 (2001).
2. J. Stano, P. Kovacs, K. Weissova, K. Micieta, K. Neubert, V. Blanarikova, and M. Korenova, *Acta Facult. Pharm. Univ. Comenianae*, **49**, 93 (2002).
3. J. Szczodrak, *Acta Biotechnol*., **19**, 235 (1999).
4. J. Rogalski and J. Lobarzewski, *Acta Biotechnol*., **15**, 211 (1995).
5. J. Stano, L. Bezakova, P. Kovacs, K. Micieta, V. Menon, and F. Andriamainty, *Pharmazie*, **52**, 569 (1997).
6. J. Poor, J. Stano, K. Neubert, J. Cizmarik, F. Andriamainty, and N. Borovkov, *Bull. Food Res*., **37**, 127 (1998).
7. S. Czigleova and D. Grancai, *Medicinal Plants*, **39**, 193 (2002).
8. K. Neubert, J. Stano, K. Micieta, P. Kovacs, and H. Tintemann, *Biol. Plant*., **45**, 307 (2002).
9. V. Gekas and M. Lopez-Leiva, *Process Biochem*., **20**, 2 (1985).
10. A. Illanes, A. Ruiz, M. E. Zung, C. Aguirre, S. Oreilly, and E. Curotto, *Bioprocess Eng*., **5**, 257 (1990).
11. M. Tomaska, P. Gemeiner, I. Marterlin, E. Sturnik, and G. Handrikova, *Biotechnol. Appl. Biochem*., **21**, 347 (1995).
12. J. Poor, J. Stano, H. Tintemann, K. Micieta, F. Andriamainty, and A. Klimecky, *Bull. Food Res*., **37**, 33 (1998).
13. J. Stano, P. Kovacs, K. Micieta, K. Neubert, H. Tintemann, and M. Korenova, *Acta Histochem*, **104**, 441 (2002).
14. G. Simons, T. Giannakouros, and J. G. Georgatsos, *Phytochemistry*, **28**, 2587 (1979).
15. T. Sawicka and A. Kacperska, *Plant. Physiol*., **145**, 357 (1995).
16. A. C. Hulst and J. Tramper, *Enzyme Microbiol. Technol*., **11**, 546 (1989).
17. P. Parascandola and V. Scardi, *O. Appl. Microbiol. Biotechnol*., **26**, 507, (1987).
18. K. Micieta, J. Stano, V. Blanarikova, E. Havranek, F. Andriamainty, N. Birjukova, S. Ignatova, and I. Safarik, *Bull. Food Res*., **38**, 153 (1999).
19. R. Hamilton, H. Pedersen, and C. K. Chin, *Biotechnol. Bioeng. Symp*., **14**, 383 (1984).
20. J. M. S. Cabral, M. M. Cadete, J. M. Novais, and J. P. Cardoso, *Ann. N.Y. Acad. Sci*., **434**, 483 (1984).
21. A. M. Golubev, J. R. B. Neto, E. V. Eneyskayta, A. V. Kulminkaya, M. A. Kerzhner, K. N. Nestcoev, and I. Polikarpov, *Acta Crystallogr*. Sect. D; *Biol. Crystallogr*. **D56**, 1058 (2000).
22. R. Hoos, A. Vasella, K. Rupitz, and S. G. Withers, *Carboh. Res*., **298**, 291 (1997).
23. K. Fukase, T. Yasukochi, Y. Suda, M. Yosida, and S. Kusumoto, *Tetrahedron Lett*., **37**, 6763 (1996).
24. E. Bedir, I. A. Khan, and L. A. Walker, *Pharmazie*, **57**, 491 (2002).
25. I. I. Mahmond, F. A. Moharram, M. S. A. Marzouk, M. W. Linsheid, and M. I. Saleh, *Pharmazie*, **57**, 494 (2002).
26. J. Stano, P. Nemec, L. Bezakova, D. Kakoniova, P. Kovacs, K. Neubert, and D. Liskova, *Acta Biochim. Polon*., **45**, 621 (1998).
27. F .Devinsky, D. Mlynarcik, I. Lacko, and L. Krasnec, *Pharmazie*, **34**, 574 (1979).
28. T. Murashige and F. Skoog, *Physiol. Plant*., **15**, 473 (1962).
29. C. L. Brown and R. H. Lawrence, *Forest. Science*, **14**, 62 (1968).
30. P. Trinder, *Ann. Clin. Biochem*., **6**, 24 (1969).
31. W. D. Kim, O. Kobayashi, S. Kaneko, Y. Sakakibara, G. G. Park, I. Kusakabe, H. Tanaka, and H. Kobayashi, *Phytochemistry*, **61**, 621 (2002).
32. M. M. Bradford, *Anal. Biochem*., **72**, 248 (1976).
33. R. A. Dixon, *Plant Cell Culture, a Practical Approach. IRL Press*, Oxford, Washington DC, (1991).