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BRIEF COMMUNICATION

Invertase in immobilized cells of *Eschscholtzia californica*

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Abstract

Cell suspension culture of *Eschscholtzia californica* Cham. were permeabilized by *Tween* 20 or 80, and immobilized by glutaraldehyde. The highest invertase activity was at pH 4.5 and temperature 50 °C. The hydrolysis of the substrate was linear for 5 h reaching 60 % conversion. The cells had high invertase activity and a good stability, and in long-term storage they showed convenient physico-mechanical properties.

Additional key words: Californian poppy, cell permeabilization, glutaraldehyde.

Immobilization techniques have a great impact on technology nowadays. In the last decades, several methods for fixation of biocatalysts have been developed. Enzymes, living or nonliving microorganisms, animal and plant cells, as well as combined systems have been bound within/or to carrier materials (Hulst and Tramper 1989, Förster 1994, Stano *et al.* 1998). Immobilization of cells or enzymes represents an effective way for obtaining highly efficient enzyme catalysts important for biotransformation processes (Klibanov 1983). Many matrices from synthetic polymers or biological materials have been used for the immobilization of cells. The most widely used technique is the application of gel particles of agar, agarose, kappa-carrageenan, collagen, chitosan, polyacrylamide, polyurethane or cellulose (Tampion and Tampion 1987, Hulst and Tramper 1989). The spontaneous adhesion or covalent binding of cells to the surfaces of insoluble carriers was also examined (Parascandola *et al.* 1987, Rogalski and Lobarzewski 1995). The use of polyvinyl-alcohol and glutaraldehyde (Wu and Wisecarver 1992, Hasal *et al.* 1992), or *Tween* and glutaraldehyde (Stano *et al.* 1998) for cell immobilization has been investigated, too.

Invertase (β-D-fructofuranosidase; EC 3.2.1.26) catalyses the hydrolyses of sucrose to glucose and fructose. The enzyme studied is used also in processes leading to mixtures of glucose and fructose (invert sugars) enabling the successive production of fructose containing preparations (Schlee and Kleber 1991, Mansfeld *et al.* 1992).

The development of new techniques of immobilization of biocatalysts is tightly connected with the progress of biotechnological processes. Because of the known fact that the cell wall slows down the transport of many compounds from and into the cell we were interested in exploring possibilities of the permeabilization of the cell wall. We assume that immobilized cells of plant origin could play in biotechnological processes a similar role as representatives of various microorganisms.

In this paper we turned our attention to the study of the effect of permeabilization on enzymatic hydrolysis of sucrose using immobilized cells of Californian poppy (*Eschscholtzia californica* Cham.).

Long term callus cultures were derived from seedlings of Californian poppy (*Eschscholtzia californica* Cham.) and continuously subcultured every 2 - 3 weeks on Murashige and Skoog (1962) medium as described by

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Andriamainty *et al.* (2000). Cell suspension was filtered through a nylon cloth and 10 g fresh mass of cells was suspended in 50 cm^3 of 0.15 M NaCl with 5 % *Tween* 20, 5 % *Tween* 80, 30 % ethanol, 50 % ethanol, 0.1 % hexadecyltrimethyl-ammonium bromide, 0.1 % hexadecylpyridinium chloride. Permeabilization proceeded for 3 h under moderate stiring at laboratory temperature. The cells were filtered off and washed with 2.5 dm^3 of distilled water and 1.5 dm^3 of 0.15 M NaCl solution. The permeabilized cells were immediately suspended in 30 cm^3 of 0.15 M NaCl and immobilized using glutaraldehyde by crosslinking as described by Poór *et al.* (1998). The immobilized cells were washed with 3 dm^3 of 0.15 M NaCl solution and separated by filtration.

Fresh and dry masses of cells in living suspension culture and in immobilized cells were determined gravimetrically. For determination of dry mass, samples were dried to constant mass at 105 °C. The effect of temperature on enzymatic activity was tested in the range from 20 to 100 °C, as described in enzyme assay (see below). The effect of glucose, fructose, cellobiose, galactose and gluconelactone on the activity of invertase in suspension cell culture and in immobilized cells was tested in 1, 5, 10 and 20 mM concentrations. The effect of pH on enzymatic activity was tested in the range from pH 4.2 to 5.2, using 0.2 M McIlvaine buffer. The immobilized cells and cell suspensions were exposed to initial glucose concentration of 200 mg dm^{-3} in the cultivation media (Murashige and Skoog 1962) without the presence of sucrose. The concentration of glucose was determined by the method of Trinder (1969). The enzyme activity was determined by a modified method of Trinder (1969) using sucrose as the substrate. The reaction mixture contained 0.1 g of wet cells, 0.4 mM sucrose in 2 cm^3 of 0.2 M McIlvaine buffer. The mixture was incubated at 30 °C for 1 h. The control contained temperature (100 °C) inactivated cells. The cells were separated from the reaction mixture, dried and enzyme activity was calculated to 1 g of dry mass. The enzyme activity was expressed in katals. Protein contents were determined by the method of Bradford (1976) using bovine serum albumin as the standard protein. The cell viability was determined by a method of Dixon (1991) with 2,3,5-triphenyltetrazoliumchloride (TTC), fluoresceindiacetate and with an oxygen electrode.

The immobilization of isolated enzymes and the permeabilization followed by immobilization are techniques widely used in technologies (Brodelius *et al.* 1979). After the immobilization of the cells using glutaraldehyde plasmolysis occurs as well as aggregation of the cells. The viability of the immobilized cells was determined by the respiratory activity measured with an oxygen electrode, and by vital staining (using 2,3,5-triphenyltetrazoliumchloride and fluoresceindiacetate) and cells immobilized by glutaraldehyde were found not viable. Also glucose was utilized only by cell suspension, but not by immobilized cells (Fig. 1).

The permeabilization of the studied cells by *Tween* 80, *Tween* 20, ethanol, hexadecylpyridinium chloride, and hexadecyltrimethylammonium bromide led to the

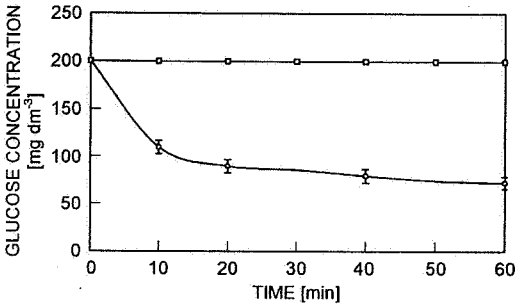

Fig. 1. Time course of glucose utilization in cells immobilized by glutaraldehyde (*squares*) and in cells in suspension (*circles*). *Vertical bars* indicate SE, $n = 5$.

leakage or degradation of proteins while the enzyme activity moderately increased with exception in ethanol used samples, and the specific activity increased in all samples tested. By glutaraldehyde crosslinking a noticable fall in the enzyme activity has been found (Table 1). The immobilized cells, like viable cells had a pH optimum of invertase at 4.5 and a minor peak of activity appeared at pH 5.3, too (Fig. 2). By cell wall permeabilization of yeasts a very significant increase of phenylalanine ammonialyase (PAL) activity was observed (Srinivasan-Nagajyothi *et al.* 1994).

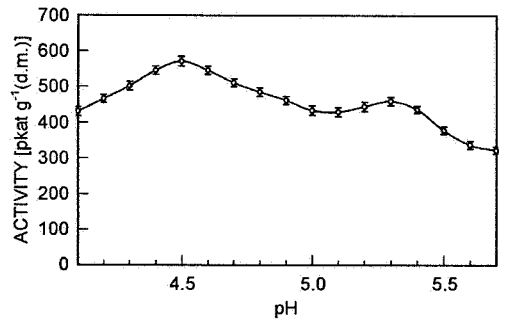

Fig. 2. pH optimum of invertase in immobilized cells of *Eschscholtzia californica*. *Vertical bars* indicate SE, $n = 5$.

By cell wall permeabilization of the suspension culture a noticable increase of invertase activity was not observed. Sucrose is probably the most widely used carbon and energy source in plant tissue culture and cell suspension (Murashige and Skoog 1962, Hamilton *et al.* 1984). In our experiment we replaced sucrose with glucose, fructose and galactose. The presence of galactose, fructose and glucose caused a decrease of the activity of this hydrolase (Table 2).

Hamilton *et al.* (1984) found that utilization of sucrose by plant tissue cultures was followed by a consistent pattern of rapid initial inversion and by the sequencial

Table 1. Protein content and invertase activity in *Eschscholtzia californica* cells in suspension, permeabilized cells by hexadecyltrimethylammonium bromide (HTAB), hexadecylpyridinium chloride (HPCH), *Tween* or ethanol and in cells immobilized by glutaraldehyde. Means of five replicates ± SE.

Cells		Protein [mg g^{-1}(d.m.)]	Activity [pkat g^{-1}(d.m.)]	Specific activity [pkat mg^{-1}(protein)]
Suspension		13.8 ± 0.22	538 ± 2.3	39.99
Permeabilized	0.1 % HTAB	9.3 ± 0.22	632 ± 3.4	67.96
	0.1 % HPCH	9.3 ± 0.21	658 ± 4.6	70.75
	5 % *Tween* 20	9.3 ± 0.22	582 ± 1.2	62.58
	5 % *Tween* 80	9.3 ± 0.20	598 ± 1.9	64.30
	30 % ethanol	9.3 ± 0.21	521 ± 3.3	56.02
	50 % ethanol	9.3 ± 0.21	514 ± 1.9	55.27
Immobilized	0.1 % HTAB	9.1 ± 0.26	521 ± 1.9	57.25
	0.1 % HPCH	9.1 ± 0.24	536 ± 1.9	58.90
	5 % *Tween* 20	9.2 ± 0.25	472 ± 3.7	51.30
	5 % *Tween* 80	9.2 ± 0.26	483 ± 1.2	52.50
	30 % ethanol	9.2 ± 0.24	427 ± 3.0	46.41
	50 % ethanol	9.2 ± 0.25	415 ± 2.2	45.11

Table 2. Influence of glucose, fructose and galactose on invertase activity [%] of immobilized cells of *Eschscholtzia californica.* Means of five replicates ± SE.

Concentration [mM]	Glucose	Fructose	Galactose
0	100	100	100
1	73 ± 0.54	66 ± 0.31	68 ± 0.31
5	70 ± 0.31	56 ± 0.44	60 ± 0.31
10	66 ± 0.31	55 ± 0.54	56 ± 0.31
20	62 ± 0.31	53 ± 0.31	53 ± 0.44

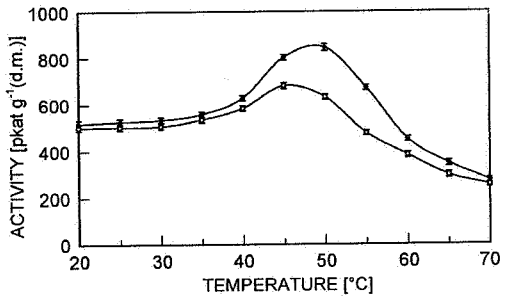

Fig. 3. Effect of temperature on activity of invertase in cells suspension (*circle*) in immobilized cells (*rhombus*) of *Eschscholtzia californica. Vertical bars* indicate ± SE, $n = 5$.

phases of glucose and fructose consumptions. Glucose and fructose are present in the media in roughly equal amounts after the first few days of inoculation, but the cells did not consume fructose until glucose was present.

Enzymatic hydrolysis of sucrose was linear for 5 h reaching 60 % of conversion, then practically stopped. The temperature optimum using immobilized cells or cell suspensions was at 50 °C and 45 °C, respectively. (Fig. 3). These values are lower compared with α-galactosidase (Stano *et al.* 1996).

The immobilized cells as well as viable cells had an apparent K_m for invertase of 4.5 mM. Similar properties were reported for invertases isolated from rice K_m = 6.6 mM (Isla *et al.* 1995), poppy K_m = 5 mM (Kováčiková 1981), maize scutellum 2.9 mM and *Schizophyllum communae* K_m = 4.8 mM (Rojo *et al.* 1994).

The inhibitory effect of *p*-chloromercuribenzoic acid in 0.1 - 0.5 mM concentration can be eliminated with 5 - 10 mM 2-mercaptoethanol, 5 - 10 mM dithiothreitol, and 5 - 10 mM cysteine, indicating that -SH groups are essential for enzyme activity (Kováčiková 1981, Machová 1994). The activity of partially purified enzyme preparations of invertase from gherkin and poppy seedlings were inhibited by glucose, galactose and fructose in a moderate way (Kováčiková 1981, Machová 1994, Liday 1981); a similar inhibitory effect was observed with immobilized cells, too (Table 2). Isla *et al.* (1995) found, that fructose is a competitive and glucose a noncompetitive inhibitor of invertase.

Invertase and other glycosidases can be perspectively applied in biotransformation processes of pharmaceutically as well as of in the food industry important compounds; their application in structure studies of these compounds is another possible field of their practical use (Grančai *et al.* 1986, Schlee and Kleber 1991, Bilisics *et al.* 1994, Bielecky and Somiari 1996, Fukase *et al.* 1996, Chopra *et al.* 2000, Vitrac *et al.* 2000,).

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