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Hydrolysis of lactose in whey permeate by immobilized β-galactosidase from *Kluyveromyces fragilis*

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Abstract

Neutral β-galactosidase from *Kluyveromyces fragilis* was immobilized on silanized porous glass modified by glutaraldehyde binding, with retention of more than 90% of its activity. Marked shifts in optimum pH (from 7.0 to 6.0) and temperature (from 35°C to 50°C) of the solid-phase enzyme were observed together with high catalytic activity and reasonable stability at wider pH and temperature ranges than those of the free enzyme. Highly efficient lactose saccharification (86–90%) in whey permeate was achieved both in a batch process and in a recycling packed-bed bioreactor.

Keywords: Yeast lactase; Whey permeate; Lactolysis; Immobilization

1. Introduction

Whey permeate, containing ca. 5% lactose, is an abundant effluent produced in cheese and casein manufacture. The biotechnological utilization of this economically valuable feedstock is largely limited by lactose due to its poor solubility, insufficient sweetness and the problem of lactose intolerance. Hydrolysis of lactose to glucose and galactose by β-galactosidase (commonly known as lactase) would overcome some of these limitations and permit greater usage of the permeate, e.g., as a substitute for corn syrup in soft drinks, fermented beverages and confectionery products [1,2].

There are basically two different ways to use β-galactosidases. The soluble enzyme is normally used for batch processes while the immobilized form lends itself to continuous operation. Despite the high cost of enzyme attachment, immobilized β-galactosidase systems remain more economically feasible than free enzyme systems, as these processes may be performed continuously and offer the possibility of reutilizing the enzyme [3–6].

The present investigation describes the best operating conditions for the hydrolysis of lactose in sweet whey permeate by both free lactase and lactase from *Kluyveromyces fragilis* immobilized on porous glass modified by glutaraldehyde.

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2. Materials and methods

2.1. Substrates and chemicals

Spray-dried whey permeate containing 85% lactose was a gift from the Institute of Food Biotechnology (Olsztyn-Kortowo, Poland). It was obtained from pasteurized raw sweet whey by ultrafiltration, demineralization and drying. Lactose, glucose, glucose oxidase, peroxidase, *o*-dianisidine, ethyl 4-hydroxybenzoate (ethyl paraben) and 3-aminopropyltriethoxysilane (APTES) were supplied by Sigma (St Louis, MO, USA). Glutaraldehyde was purchased from Merck (Darmstadt, Germany). All other products used were of reagent or analytical grade.

2.2. Enzyme source and assay

Stock cultures of *K. fragilis* IPF-1, maintained at 4°C on 2% malt agar slants, were used for inoculations. The lactase production medium at pH 5.0 [7] in 500-ml conical flasks, each with 100 ml of the medium, supported by 2.5% lactose, 0.25% urea, and 0.25% yeast extract was autoclaved for 20 min at 117°C and inoculated with 5% (v/v) of the 24-h seed culture, previously incubated with shaking in the same medium. The submerged culture was run at 30°C for 12 h on a rotary shaker at 220 rpm.

For the preparation of cell-free extract containing intracellular lactase, 0.05 M K-phosphate buffer (pH 7.0) with 0.1 mM $MnCl_2$ was used as the extraction medium. The cells were harvested by centrifugation at 4000 rpm for 10 min. The thoroughly buffer-washed yeast cake was then disrupted for 10 min at 4°C in a mortar with pestle using equal weight of corundum A-240 (Polwid II, Cieszyn, Poland) as an abrasive material. The homogenate was centrifuged at 14 000 rpm for 15 min. The sediment was removed, and the remaining volume was made up with buffer to 10 ml. The crude lactase was then purified (about 16 times, the yield is 48%) by the one-step affinity chromatographic method [8], in which epoxy(bis-oxirane)-silanized porous glass as a support and lactose as a ligand were applied. The diluted preparation with a β-galactosidase activity of 5 U/ml was used for lactose hydrolysis (lactolysis). The transgalactosylase activity was relatively low: oligosaccharide formation was limited to ca. 5–10% of the total sugars present.

The lactase activity was determined with a standard assay by mixing 0.5 ml of enzyme slurry (50 mg, wet) or solution with 0.5 ml of 1% lactose in 0.05 M K-phosphate buffer (pH 7.0 for the free enzyme, and pH 6.0 for the immobilized one), containing 0.1 mM $MnCl_2$. It was then followed by incubation at 35°C (for the free enzyme) or 50°C (for the immobilized one) for 30 min, and measuring the glucose released with a glucose oxidase–peroxidase reagent [9]. One unit of the enzyme activity (U) was defined as the amount producing 1 μmol glucose/min under the described conditions; 1 U corresponds to 16.67 nkat.

2.3. Immobilization of lactase on controlled porous glass (CPG)

CPG (Cormay, Lublin, Poland) with a boron-enriched surface was prepared according to the method described earlier [10]. The specific surface area, S (m^2/g), average pore diameter, D (nm) and average pore volume V (cm^3/g) were 82, 40 and 1.26, respectively. The glass was activated by APTES following the method which allows a high-density level of the amino groups on the glass surface to be obtained [11]. The purified lactase was then covalently linked to silanized glass (APTES-CPG) via amino groups, using glutaraldehyde as described by Lappi et al. [12]. The carrier-bound enzyme was thoroughly washed with distilled water and kept under water at 4°C until further use. The yield of lactase immobilization was estimated on the basis of the difference between the activity (or protein amount) added to the glass and that recovered in the pooled supernatant and washing fractions. The coupling efficiency was very high since more than 90% of

the applied enzyme was active and 85.7% of the protein was bound to the support.

2.4. Effect of the pH value and temperature on the activity and stability of free and immobilized lactase

These effects were studied by the standard assay described under Section 2.2. The influence of pH on the activity of lactase was examined in the range of 5.5–8.0. The pH stability was determined by preincubation of the enzyme at 30°C for 12 h, followed by activity measurement at optimum pH. The effect of temperature on activity was measured at the optimum pH of 7.0 from 30°C to 60°C. Thermal stability of the enzyme was determined by incubation without lactose for 1 h at optimum pH and measuring the residual activity at optimum temperature. The relative activity at each pH and temperature is expressed as the percentage at which the enzyme reached its maximum activity.

2.5. Hydrolysis of whey permeate lactose by free and immobilized β-galactosidase

2.5.1. Batch and repeated batch hydrolysis

The standard hydrolysis was conducted in plugged round-bottomed flasks (50 ml) in the presence of 0.1% ethyl 4-hydroxybenzoate (ethyl paraben). Unless otherwise stated, the whey permeate (pH 7.0, 20 ml) contained 5% of lactose and 5 U/g substrate of *K. fragilis* β-galactosidase. The flasks were incubated for 48 h at 35°C in a water bath shaker and agitated at 150 rpm. In the case of immobilized lactase, the whey permeate (pH 6.0) containing 5% of lactose and 4.6 U/g substrate of the enzyme was incubated for 20 days at 45°C in a water bath shaker at 150 rpm. The reaction mixture was replaced every 48 h and the carrier-bound enzyme was washed off with water before being transferred into the fresh medium. Samples were withdrawn periodically and analyzed enzymatically for glucose.

2.5.2. Column hydrolysis

Three grams (wet mass) of lactase immobilized on APTES-CPG (4.6 U/g lactose) was packed under gravitational conditions in a column (1.5×3.5 cm), equilibrated with 0.05 M K-phosphate buffer (pH 6.0) containing 0.1 mM $MnCl_2$. A solution of whey permeate (lactose 5%, pH 6.0, 100 ml) was then recycled through the column at 45°C for 48 h at various flow-rates (0.3–2.0 ml/min). Ethyl paraben (0.1%) was added to the whey permeate solutions to prevent the growth of microorganisms. Samples of the column effluent were taken at regular intervals, and the eluted glucose was determined in duplicate enzymatically (see lactase assay).

The detailed experimental conditions are described in the subsequent figures and tables. The percentage of lactolysis was calculated using the following equation: saccharification (%) = glucose formed (mg) $\times$ 2 $\times$ 0.95 $\times$ 100/lactose (mg). All data given here are averages of the three replicate experiments. The mean standard error of the lactose saccharification was $\pm$1.35% and ranged from $\pm$0.04 to 2.52.

2.6. Other analyses

Lactose and oligosaccharide contents in powdered whey permeate were determined by high-performance liquid chromatography, as described previously [13,14]. Protein was analyzed using the Schacterle and Pollack method [15]. All analyses were done at least in duplicate and the data given are the averages of all the measurements.

3. Results and discussion

3.1. Optimization of the hydrolysis process

The saccharification of whey permeate by *K. fragilis* β-galactosidase was studied as a function of reaction time, substrate and enzyme concentration, pH and temperature (Table 1).

Table 1
Optimization of the enzymatic hydrolysis of lactose in whey permeate

Experiment	Factor varied	Saccharification (%) (after a 48-h hydrolysis[a,b,c,d])
1	Reaction time (h)[e]	
	12	24.8
	24	56.0
	48	69.1
	72	68.1
2	Substrate concentration (%)[a]	
	1.0	47.7
	2.5	58.7
	5.0	70.5
	10.0	48.5
	20.0	25.7
3	Lactase concentration (U/g lactose)[b]	
	1	70.9
	5	86.0
	10	85.1
	15	83.6
4	pH[c]	
	5.5	20.5
	6.0	60.7
	6.5	84.9
	7.0	94.8
	7.5	67.9
	8.0	15.5
5	Temperature (°C)[d]	
	30	72.8
	35	96.1
	40	90.2
	45	67.3
	50	24.2

[a] *K. fragilis* lactase, 1 U/g lactose; 35°C; pH 6.5.
[b] Whey permeate (lactose, 5%); 35°C; pH 6.5.
[c] Whey permeate (lactose, 5%); *K. fragilis* lactase, 5 U/g lactose 35°C.
[d] Whey permeate (lactose, 5%); *K. fragilis* lactase, 5 U/g lactose; pH 7.0.
[e] Whey permeate (lactose, 5%); *K. fragilis* lactase, 1 U/g lactose; pH 6.5; 35°C.

The results show that whey permeate containing 5% of lactose and 5 U lactase/g substrate (temperature at 35°C, pH 7.0 and a 48-h hydrolysis time) may be considered as the optimum conditions for lactolysis, resulting in a 96% lactose conversion.

3.2. Immobilization of β-galactosidase and its properties

The optimum pH of the immobilized enzyme was displaced (by 1.0 pH unit) towards the slightly acidic region (pH 6.0), and showed a more widely spread pH profile as compared to the free lactase (Fig. 1). Also, stability of the immobilized enzyme at low pH is much better (Fig. 2). These properties are very useful for lactolysis in sweet whey permeate, which has a pH range of 5.5–6.0. Such displacements of optimum pH values on enzyme immobilization are well known, including commercial β-galactosidases [5,16,17], and may be brought about by the partitioning of protons, affected by ionized groups in the matrix [18] or the effect of electrostatic potential of the polyelectrolyte carrier on the local concentration of protons [19].

Immobilization increases the optimum temperature from 35°C to 50°C, including broadening of the temperature profile (Fig. 3). In addition, thermal stability of the immobilized lactase has increased up to 50°C (Fig. 4). Thus, a higher stability of the immobilized lactase on porous glass beads at lower pH and higher temperature makes this form more convenient and more useful for lactose hydrolysis in the recycling process, also preventing the whole process from oligosaccharide formation [20] and microbial growth in the reactor. Previously, sim-

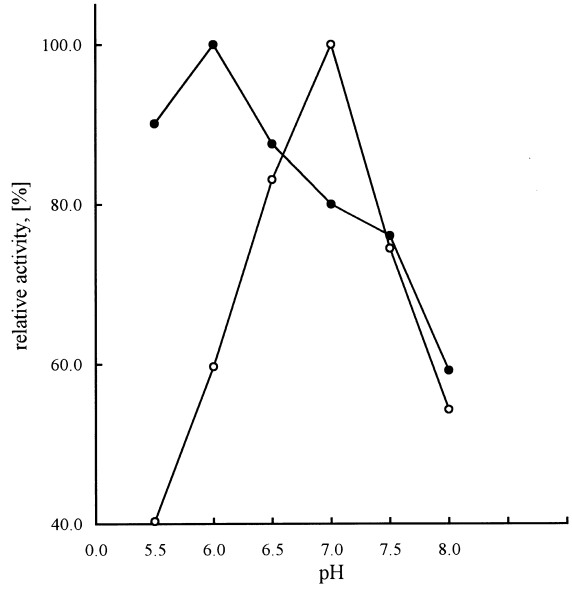

Fig. 1. The pH/activity profiles for native *K. fragilis* lactase (-○-) and lactase immobilized on alkylamine glass (-●-).

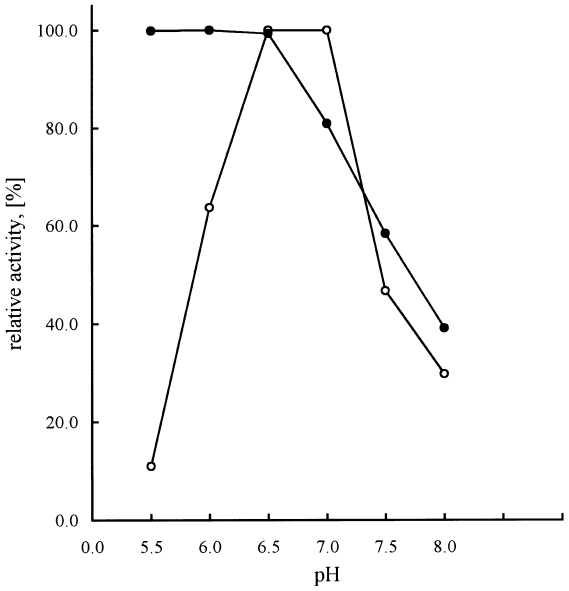

Fig. 2. The pH stability of native *K. fragilis* lactase (-○-) and lactase immobilized on alkylamine glass (-●-).

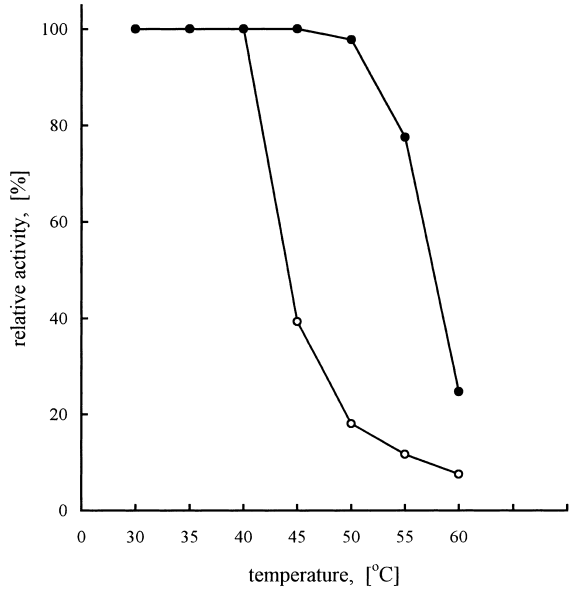

Fig. 4. Thermal stability of native *K. fragilis* lactase (-○-) and lactase immobilized on alkylamine glass (-●-).

ilar effects have been observed, e.g., for pectin lyase [21], catalase [22] or β-galactosidase [5,16].

3.3. Batch and column hydrolysis of whey permeate by immobilized β-galactosidase

Repeated batch whey permeate hydrolysis by immobilized lactase allowed up to five 48-h

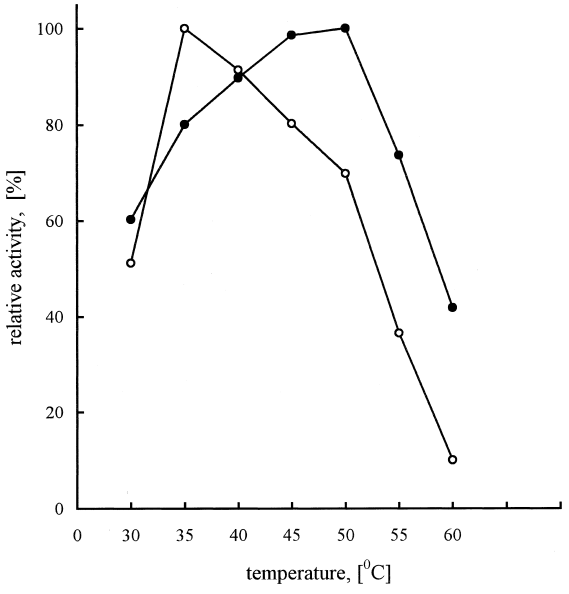

Fig. 3. Temperature/activity profiles for native *K. fragilis* lactase (-○-) and lactase immobilized on alkylamine glass (-●-).

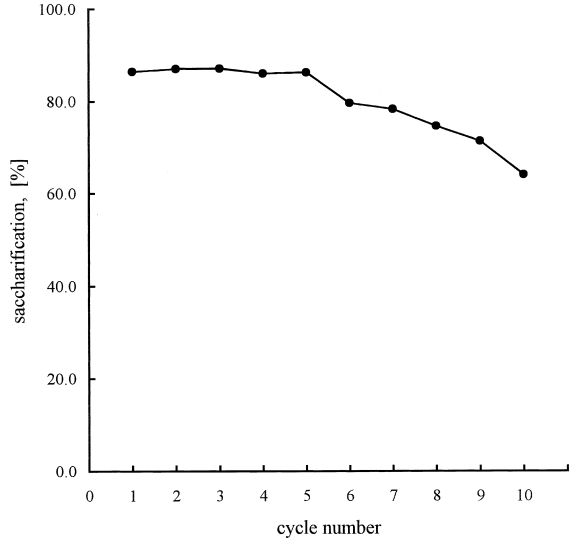

Fig. 5. Repeated batch hydrolysis of lactose in whey permeate using *K. fragilis* lactase immobilized on alkylamine glass. The hydrolysis was performed in shake flasks in 48-h cycles.

Table 2
Saccharification of lactose by recycling a solution of whey permeate through a column of immobilized lactase for 48 h[a]

Time (h)	Flow rate (ml/min)	Residence time[b] (min)	Saccharification (%)	Percent saccharification/ residence time (min)
2	2.0	3.1	33.4	10.8
6	2.0	3.1	58.4	18.8
12	2.0	3.1	66.5	21.5
24	2.0	3.1	69.2	22.3
48	2.0	3.1	82.6	26.6
2	0.7	8.8	36.7	4.2
6	0.7	8.8	59.5	6.8
12	0.7	8.8	67.8	7.7
24	0.7	8.8	70.3	8.0
48	0.7	8.8	87.3	9.9
2	0.3	20.6	25.8	1.3
4	0.3	20.6	40.2	2.0
12	0.3	20.6	69.2	3.4
24	0.3	20.6	73.8	3.6
48	0.3	20.6	90.0	4.4

[a]Column hydrolysis was performed by the coupling of *K. fragilis* lactase at various flow rates to porous glass. The other experimental details are as described in Section 2.

[b]The residence time is the time during which the liquid is flowing through the column; i.e., residence time = packed column volume (ml)/flow rate (ml/min) = 6.185 ml/flow rate.

cycles without any notable decrease in lactose saccharification (Fig. 5).

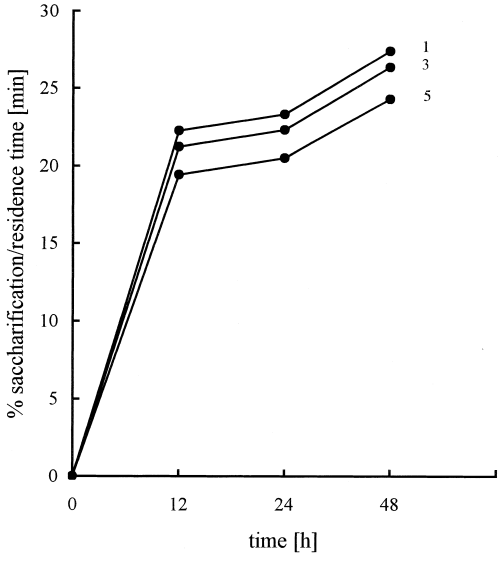

Fig. 6. Effect of time on the ratio (saccharification/residence time) on passage of a solution of whey permeate through a column of immobilized lactase. Hydrolysis was performed in 48-h cycles at a flow rate of 2 ml/min using *K. fragilis* lactase immobilized on alkylamine glass. The numbers in the particular curves represent the successive cycles of hydrolysis.

Column whey permeate hydrolysis by the immobilized enzyme in a recycling system shows conversions up to 90% (Table 2). Similar results were obtained for lactase from *Kluyveromyces lactis* immobilized on thiol-reactive gels [23], for β-galactosidase from *Aspergillus niger* immobilized on diazotized porous glass [24], for *A. oryzae* β-galactosidase covalently attached to a PVC-silica sheet [25], and other immobilized β-galactosidase preparations applied in whey permeate hydrolysis [26–30]. A relatively constant ratio (percent saccharification/residence time) obtained in successive cycles of hydrolysis (Fig. 6) gives an indication that the packed column was catalytically active over the whole of the hydrolysis period. Thus, the readiness with which reasonable flow rates were obtained was encouraging and suggests that further applications might be practicable.

4. Conclusions

The immobilization of *K. fragilis* lactase on porous glass beads is a convenient and an inex-

pensive method giving an enzyme preparation of good operational stability and activity (low pH, high temperature) for sweet whey, sweet whey permeate or other dairy products hydrolysis, both in a batch and in a recycling column reactor. Porous glass matrices with various degrees of controlled porosity for a reactor-separator with the immobilized biocatalyst are currently under investigation.

Acknowledgements

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