Production of Lactase by Candida pseudotropicalis Grown in Whey

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Lactase (β-D-galactosidase) was produced by Candida pseudotropicalis grown in deproteinized whey. Maximum enzyme production in 2% whey was obtained by supplementation with 0.15% yeast extract, 0.1% (NH₄)₂SO₄, and 0.05% KH₂PO₄ (wt/vol). Highest enzyme values (4.35 U/mg of cells and 68 U/ml) were obtained with 10 to 12% whey, while enzyme yield was maximal in 2% whey (0.87 U/mg of whey). Optimal initial pH for cultivation was 3.5. The best conditions for extraction included 2% (wt/vol) chloroform, 10 h of treatment, pH 6.6 and higher, and 30 to 37°C. Optimum pH and temperature for enzyme activity were 6.2 and 47°C. The enzyme had a Kₘ for O-nitrophenyl-β-D-galactopyranoside of 3.06 × 10⁻⁵ M and the initial Vₘₐₓ was estimated as 6.63 × 10⁻⁵ M per min. It hydrolyzed 50 and 100% of the lactose in whey and milk within 4 and 5 h, respectively, at 37°C. The lyophilized enzyme retained 95% of activity for 3 months when stored at −20°C.

Treatment of milk and milk products with lactase (β-D-galactosidase, EC 3.2.1.23) to reduce their lactose content seems to be an appropriate method to increase their potential uses (12, 22, 23, 28) and to deal with the problems of lactose insolubility and lack of sweetness. Furthermore, this treatment could make milk, a most valuable food, available to a large number of adults and children intolerant to lactose (1, 7, 9, 15, 24, 25). Preparations of lactase have been obtained from several microorganisms (3, 13, 20, 27, 30); among these, the lactose-fermenting yeasts are considered good sources of the enzyme (2, 6, 18, 19, 31, 32). One of these yeasts, Candida pseudotropicalis, has been pointed out as having a good lactase potential (11, 33). The present study deals with the determination of nutrient requirements, growth conditions, and extraction conditions for maximal lactase production by C. pseudotropicalis grown in whey. Some properties of the enzyme were determined, and the lactase preparation was tested for its ability to hydrolyze lactose in milk and whey.

MATERIALS AND METHODS

Organism. C. pseudotropicalis NCYC 744 was obtained from the National Collection of Yeast Cultures, Surrey, England.

The yeast was maintained at room temperature with monthly transfers in solid medium containing (wt/vol) 0.3% malt extract (Difco), 0.3% yeast extract (Difco), 0.5% peptone (Difco), 2% lactose (Sigma), and 2% agar (Baltimore Biological Laboratory).

Media. Sweet, dried whey was obtained from Industrias Lácteas Torondoy, Caja Seca, Edo. Zulia, Venezuela. It contained (wt/wt) 73% lactose, 10.8% protein, and 0.75% phosphorus (4).

Deproteinized whey solutions were prepared as described previously (4) and supplemented with yeast extract, (NH₄)₂SO₄, and KH₂PO₄, as required, and the pH was adjusted as needed with 2 M H₂SO₄ and 2 N KOH.

Propagatión. The cultures were grown in cotton-plugged, 300-ml flasks containing 45 ml of medium plus 5 ml of a 12-h-old inoculum and incubated in a constant-temperature reciprocating shaker bath (American Optical) at 120 strokes per min with 3.7 cm of amplitude. The temperature was maintained at 30°C.

Cell concentrations were estimated by measuring absorbance in a Klett-Summerson colorimeter (no. 54 filter, 530 to 580 nm) and relating the readings to biomass dry weight with a calibration curve.

The cells were harvested at 7,000 × g for 5 min at 4°C.

Lactase production. Enzyme production was studied as a function of medium supplementation with yeast extract (0.01 to 0.2%), (NH₄)₂SO₄ (0.05 to 0.5%), and KH₂PO₄ (0.01 to 0.4%), initial pH of the media, and whey concentration (2 to 14%).

Enzyme extraction. The method used to extract the enzyme was that of Mahoney et al. (18) with some modifications. The cells were washed and resuspended in 0.1 M potassium phosphate buffer containing 0.5 mM MgSO₄ and 0.1 mM MnCl₂ (supplemented phosphate buffer). The effects of chloroform concentration (1 to 4%, vol/vol), temperature (23 to 45°C), pH (5.8 to 7.7), and time of treatment (5 to 30 h) on enzyme yields were determined.

The extraction mixtures were centrifuged at 7,000 × g for 5 min at 4°C, and the supernatants were...
assayed for lactase activity. For further concentration of the enzyme from a crude extract, the mixture was centrifuged at 11,000 \( \times g \) for 30 min at 4°C, and the supernatant was treated with 1 volume of acetone at 4°C. The precipitate formed was collected by centrifugation at 6,000 \( \times g \) for 15 min at 4°C, resuspended, dialyzed against distilled water, and lyophilized. The enzyme preparation was divided into three portions and stored at -20°C, 5°C, and room temperature (-26°C) to test stability of the enzyme at weekly intervals.

The protein content of the material was determined by the method of Lowry et al. (17).

**Assay of enzyme activity.** The assays were carried out at 37°C and performed as described by Mahoney et al. (18) by utilizing as substrate 1.25 mM \( o \)-nitrophenyl-\( \beta \)-D-galactopyranoside (ONPG, Sigma) in 0.1 mM supplemented phosphate buffer at pH 6.6. A unit of enzyme activity was defined as the amount of enzyme required to liberate 1 \( \mu \)M of \( o \)-nitrophenol (ONP) in 1 min. The activity of lactase was also determined with reconstituted whole powder milk at a concentration of 130 g/liter (4.5 to 4.9%, wt/vol, lactose) and milk whey at 20 g/liter (1.2 to 1.5%, wt/vol, lactose) as substrates. The mixtures contained 0.1 ml of enzyme suspension (0.01 to 0.05 mg of protein per ml) per 0.9 ml of milk or whey. The released glucose was estimated with glucose oxidase by the method of Raabo and Terkildsen (26) as modified by Sigma (Technical Bulletin no. 510).

**Analyzes.** Lactose as total sugars was estimated by the anthrone method of Scott and Melvin (29).

**Phosphorus determinations.** Phosphorus determinations were performed by the method of Chen et al. (5).

**Enzyme properties.** Optimal pH and temperature for lactase activity were determined with mixtures containing 0.1 ml of enzyme suspension and 4 ml of 1.25 mM ONPG in 0.1 M supplemented phosphate buffer.

Heat stability of lactase was determined at 51 and 56°C with suspensions containing 1 mg of enzyme preparation per ml in 0.1 M supplemented potassium phosphate buffer at pH 6.2. Samples (0.1 ml) were removed at 1-min intervals and assayed for residual activity. The Michaelis constant (\( K_m \)) was determined by the method of Lineweaver and Burk (16) with ONPG as substrate.

**RESULTS**

**Concentrations of substrates.** The effects of yeast extract, \( (NH_4)_2SO_4 \), and \( KH_2PO_4 \) on cell growth and lactase yields were examined. The concentrations of whey (2%) and two of the additives were kept constant, while that of the third was varied. After 24 h of cultivation, cell concentrations, remaining lactose, and lactase activities were measured.

Maximum cell concentration and yields were obtained with 0.15% yeast extract; although 0.05% was sufficient to produce over 90% lactose consumption, highest enzyme levels in cells were observed upon addition of 0.1% or more yeast extract. Total lactase activity (U/ml) increased with increasing concentrations of yeast extract (Table 1). No significant differences in lactose utilization and biomass production were observed with different concentrations of \( (NH_4)_2SO_4 \) or \( KH_2PO_4 \) (data not shown). However, the enzyme levels in the cells, as well as total activity, were maximal when \( (NH_4)_2SO_4 \) was added at 0.1% or higher and \( KH_2PO_4 \) was added at 0.05%; at higher concentrations of the latter, the enzyme values decreased (Table 1).

Biomass production increased with increasing concentrations of whey up to 12%, while lactose utilization and cell yields decreased. Lactase activity per cell and per milliliter was highest with 10% whey and decreased for more concentrated whey (Fig. 1). The highest enzyme yield (0.87 U/mg of whey) was obtained with 2% whey.

**pH of medium.** Maximal production of lactase was observed when the initial pH of the medium was adjusted to 3.5.

**Enzyme extraction conditions.** Chloroform at a concentration range from 1 to 4% was used to treat yeast suspensions from 5 to 30 h. Best results were obtained by treatment with 2% chloroform for 10 h.

Maximal extraction was obtained between pH values of 6.6 and 7.8; higher pH values were not investigated. Optimum temperatures for extraction were 30 to 37°C (data not shown).

**Enzyme properties.** The effect of pH on the activity of lactase was studied by utilizing supplemented phosphate buffer at pH values from 5.8 to 7.7. Peak activity was observed at pH 6.2.

The effect of temperature was examined within a range of 24 to 56°C. At 45°C the enzyme was denatured, but the enzyme retained 90% of its activity at 56°C (data not shown).

**Table 1.** Effect of yeast extract, \( (NH_4)_2SO_4 \), and \( KH_2PO_4 \) on lactase production by C. pseudotropicalis grown on 2% deproteinized whey.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Conc (%, wt/vol)</th>
<th>Lactase activity (U/mg of cells)</th>
<th>Total lactase activity (U/ml)</th>
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<tbody>
<tr>
<td>Yeast extract</td>
<td>0</td>
<td>1.13</td>
<td>5.39</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>1.56</td>
<td>8.97</td>
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<tr>
<td></td>
<td>0.05</td>
<td>2.08</td>
<td>14.06</td>
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<tr>
<td></td>
<td>0.1</td>
<td>2.21</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>2.01</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>2.27</td>
<td>19.82</td>
</tr>
<tr>
<td>( (NH_4)_2SO_4 )</td>
<td>0</td>
<td>0.79</td>
<td>5.03</td>
</tr>
<tr>
<td></td>
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<td>1.20</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>2.68</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>2.60</td>
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<tr>
<td>( KH_2PO_4 )</td>
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<td>1.48</td>
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<td></td>
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</tr>
<tr>
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<td>1.52</td>
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</tr>
<tr>
<td></td>
<td>0.4</td>
<td>1.59</td>
<td>14</td>
</tr>
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</table>
possessed maximal activity. The enzyme was totally inactivated within 4 min when heated at 56°C, while it retained 14% of the original activity after 16 min at 51°C (Fig. 2). The lyophilized enzyme remained stable for 3 months when stored at -20°C, retaining over 95% of the original activity. At 5 and 26°C, 90% of the activity was maintained for 3 and 2 weeks, respectively (Fig. 3).

With ONPG as substrate, a value of $3.06 \times 10^{-3}$ M was obtained for the Michaelis constant ($K_m$), and the initial $V_{max}$ was estimated as $6.63 \times 10^{-8}$ M/min.

Lactase hydrolysis in milk and whey. Lactase added at a concentration of 0.05 mg of protein per ml to 2% whey hydrolyzed 50% of the available lactose at 37°C within 4 to 5 h. Under the same conditions (0.05 mg of protein per ml of milk and 37°C), over 98% of the lactose in milk was converted to monosaccharides within 5 h (Fig. 4).

**DISCUSSION**

Increases in lactase production, lactose consumption, and cell yields were observed upon addition of yeast extract to whey media. The yeast extract may be supplying the vitamins required by *C. pseudotropicalis* NCYC 744. This yeast has been found to require nicotinic acid, calcium pantothenate, biotin, thiamin, and pyr-
Lactase hydrolysis in whey (○) and milk (●) by C. pseudotropicalis lactase at 37°C.

Lactose conversion was more efficient in milk than in whey. Total hydrolysis of the sugar in milk at 37°C was obtained within 5 h. These results compared to those obtained with a commercial S. lactis lactase preparation (14).

The results presented here indicate that C. pseudotropicalis grown in whey may represent a good source for the production of commercial lactase.

Further characterization of the enzyme is in progress.

ACKNOWLEDGMENTS

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LITERATURE CITED


