

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% $(\text{NH}_4)_2\text{SO}_4$, and 0.05% KH_2PO_4 (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 K_m for *O*-nitrophenyl- β -D-galactopyranoside of 3.06×10^{-3} M and the initial V_{max} was estimated as 6.63×10^{-8} 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 In-

dustrias 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, $(\text{NH}_4)_2\text{SO}_4$, and KH_2PO_4 , as required, and the pH was adjusted as needed with 2 M H_2SO_4 and 2 N KOH.

Propagation. 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, 520 to 580 nm) and relating the readings to biomass dry weight with a calibration curve.

The cells were harvested at $7,000 \times g$ 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%), $(\text{NH}_4)_2\text{SO}_4$ (0.05 to 0.5%), and KH_2PO_4 (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 MgSO_4 and 0.1 mM MnCl_2 (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 $7,000 \times g$ 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 ($\sim 26^\circ\text{C}$) 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- β -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 μM 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).

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

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 mM 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, $(\text{NH}_4)_2\text{SO}_4$, 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 $(\text{NH}_4)_2\text{SO}_4$ or KH_2PO_4 (data not shown). However, the enzyme levels in the cells, as well as total activity, were maximal when $(\text{NH}_4)_2\text{SO}_4$ 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

TABLE 1. Effect of yeast extract, $(\text{NH}_4)_2\text{SO}_4$, and KH_2PO_4 on lactase production by *C. pseudotropicalis* grown on 2% deproteinized whey

Supplement	Concn (%, wt/vol)	Lactase activity (U/mg of cells)	Total lactase activity (U/ml)
Yeast extract	0	1.13	5.39
	0.01	1.56	8.97
	0.05	2.08	14.06
	0.1	2.21	14.9
	0.15	2.01	17.6
	0.2	2.27	19.82
$(\text{NH}_4)_2\text{SO}_4$	0	0.79	5.03
	0.05	1.20	9.3
	0.1	2.68	18.6
	0.25	2.68	17.9
	0.5	2.60	17.4
KH_2PO_4	0	1.48	12.93
	0.025	1.76	15.5
	0.05	1.80	15.95
	0.1	1.68	14.8
	0.2	1.52	13.4
	0.4	1.59	14

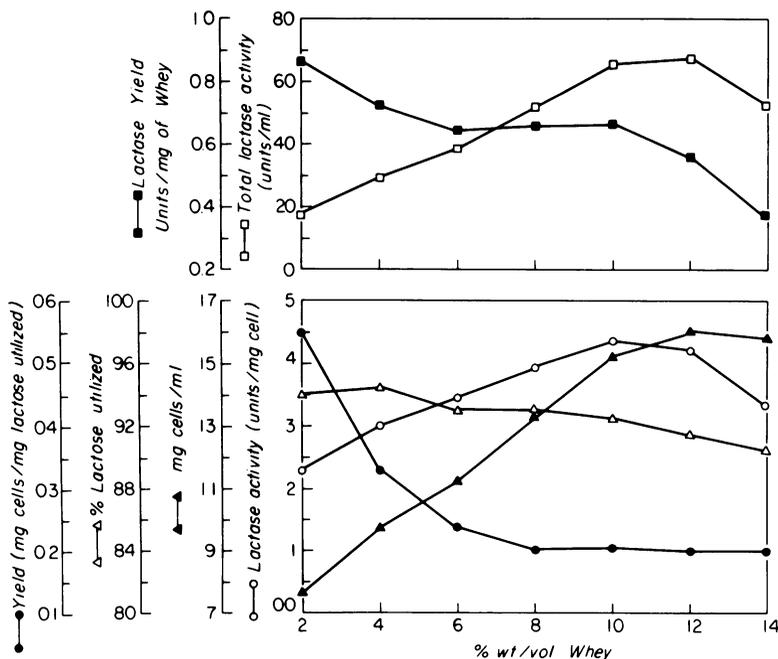


FIG. 1. Effect of whey concentration on cell growth, lactose utilization, and enzyme production.

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 3.06×10^{-3} M was obtained for the Michaelis constant (K_m), and the initial V_{max} was estimated as 6.63×10^{-8} M/min.

Lactose 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-

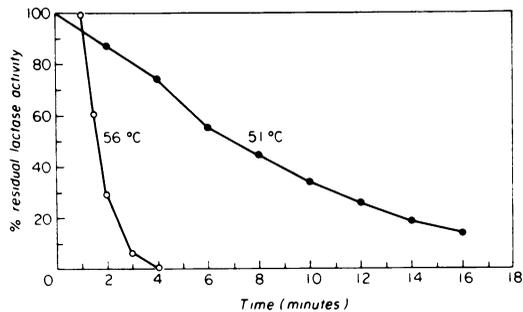


FIG. 2. Thermal inactivation of lactase in solution.

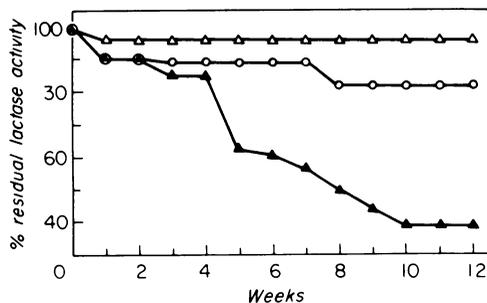


FIG. 3. Stability of the lyophilized enzyme under different storage temperatures: Δ , -20°C; \circ , 5°C; and \blacktriangle , 26°C.

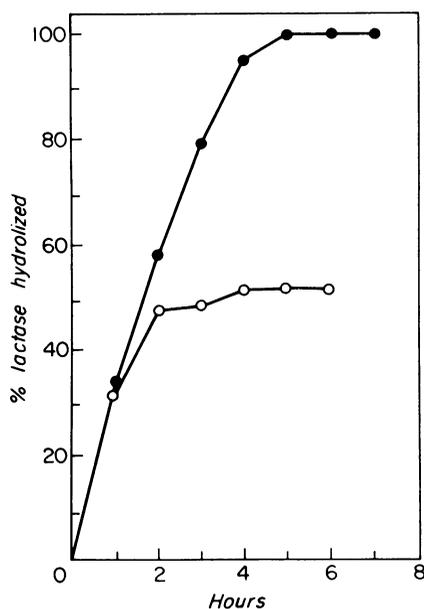


FIG. 4. Lactose hydrolysis in whey (○) and milk (●) by *C. pseudotropicalis* lactase at 37°C.

idoxine (F. J. Castillo and S. B. de Sánchez, *Acta Cient. Venez.* 28:119–120, 1977). The stimulating effect of growth factors in the production of lactase in other microorganisms has been reported (27, 32). However, Mahoney et al. (18) detected no significant changes in lactase production by *Kluyveromyces fragilis* grown in whey, with or without supplementation with yeast extract or corn steep liquor. This discrepancy could be due to differences in the vitamin content of the whey media utilized which, in turn, may reflect differences in processing treatments as suggested by Glass and Hedrick (10).

Although the supplementation of whey with nitrogen and phosphorus had no significant effect on lactose utilization and cell yields, some stimulation in lactase production was observed with the addition of the salts. Similar results have been obtained with *Streptococcus thermophilus* (27) and *Saccharomyces fragilis* (32). A fall in enzyme production was observed when KH_2PO_4 was added at concentrations higher than 0.05%; similar observations with *S. thermophilus* upon addition of other phosphorus salts have been made (27). Although the highest enzyme yield (0.87 U/mg of whey) was obtained with 2% whey, production of lactase, per milligram of cells and per milliliter, was maximum in 10 to 12% whey (7.3 to 8.7% lactose), in contrast with the 10 to 15% lactose reported as optimal for *S. fragilis* (18). Mahoney et al. (18) reported a maximal lactose yield of 15 U/ml for *K. fragilis* grown in whey, containing 15% lactose, in shake

flasks. With *C. pseudotropicalis* in 12% whey (8.7% lactose), 67.5 U/ml were obtained here. The optimal initial pH of 3.5 found for *C. pseudotropicalis* is lower than the 4.5 of *S. fragilis* (32) and 6.5 to 7.4 for *S. thermophilus* (27). Low pH is desirable for it will reduce the possibility of contamination as shown by Wendorff et al. (32).

Conditions for maximal lactase extraction from fresh cells in phosphate buffer were 30 to 37°C, pH 7, 2% chloroform, and 10 h of treatment. Similar results were reported for *S. fragilis* (18). Optimum pH and temperature for lactase activity, with ONPG as substrate, were within the range of values reported previously for *C. pseudotropicalis* and other microorganisms (2, 6, 13, 21, 27, 31, 33).

Activity of the lyophilized enzyme remained at over 95% when stored at -20°C . These seem to be the most suitable conditions for storage because lactase preparations have a tendency to lose activity when in solution or when kept at higher temperatures (6, 18, 20, 31). The Michaelis constant (K_m) calculated here for the lactase of *C. pseudotropicalis* NCYC 744 was slightly higher than those of *S. fragilis* NRRL-Y-1109 (31) and 189-K-S (8), *Saccharomyces lactis* (2), and *Aspergillus foetidus* (3), and lower than *E. coli* and yeast (21), *Aspergillus niger* (34), and *Actinomyces viscosus* (13).

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 compare 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.

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