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EXTRACYTOPLASMIC PHOSPHATASES OF SELECTED SPECIES OF *SACCHAROMYCES, KLUYVEROMYCES* AND *RHODOTORULA*

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Key Word Index—*Rhodotorula*; *Saccharomyces*; *Kluyveromyces*; yeast; extracytoplasmic phosphatases.

Abstract—Phosphatase activities of yeasts belonging to the genera *Saccharomyces*, *Kluyveromyces* and *Rhodotorula* were studied. *Rhodotorula rubra* exhibited activities at acid, neutral and alkaline pH; the other yeasts only had activity at acid pH. Growing yeasts in a constant pH (4.5) medium decreased phosphatase activities in *Saccharomyces* and *Kluyveromyces*, while neutral activity was enhanced in *Rhodotorula rubra* which excreted more enzyme under these conditions. Washing cells with sucrose solutions lowered phosphatase activities in all yeasts, due to enzyme liberation. Acid phosphatase activities in isolated and purified cell walls were very small. Phosphatases thus appear not to be strongly bound to yeast cell walls.

INTRODUCTION

The study of *Rhodotorula rubra* revealed three phosphatase activities: acid, neutral and alkaline [1]. In *Saccharomyces* species, only an acid phosphatase activity could be observed [2, 3]. These phosphatases are supposed to be located in the cell wall [2, 4–7], or in the periplasmic space [8, 9]. Results obtained in this field are hardly comparable since they have been obtained with different species cultured in different media. However, the differences in the activities do not seem to be due to an inhibition of enzyme synthesis, but to the variable degree of fixation to the cell wall. Cell wall permeability may be dependent on the pH of the culture medium [10].

The aim of the present study was: (a) to compare phosphatase activities in three yeasts cultured under the same conditions; and (b) to study the variations of the phosphatase activities in the presence of factors which may alter the integrity of the cell wall.

RESULTS

Growth of yeasts in variable or constant pH media

Grown in medium D with varying pH, the seven strains *S. cerevisiae* 4 and 183, *Rh. rubra* RMP and Rjl, *Kl. lactis*, *Kl. fragilis* and *Kl. bulgaricus* had a similar growth for the first 24 hr, lag phase of 4 hr, generation time of 1.30 after 18 hr. There was a drop in pH from 5.6 to 3.5, even to 3.0 during the first 48 hr, then an increase (Fig. 1).

The growth curves of yeasts grown at a constant pH were similar to those of yeasts grown at variable pH, so a constant pH had no influence on cell development. For the study of phosphatase activity, cells sampled after 18 hr growth were used, so they were in full log phase. After staining with methylene blue, 14.6% of the cells were coloured, hence more than 80% of the cells were alive.

Phosphatase activity of whole cells

Qualitative and quantitative activities were determined on cells harvested under the same conditions. After

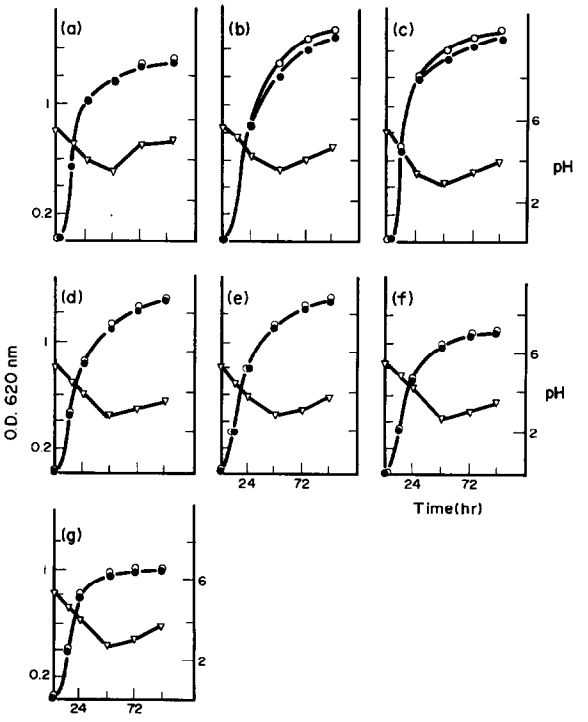

Fig. 1. Growth curves of (a) *Kl. bulgaricus*, (b) *Kl. fragilis*, (c) *Kl. lactis*, (d) *Rh. rubra* RMP, (e) *Rh. rubra* Rjl, (f) *S. cerevisiae* 4 and (g) *S. cerevisiae* 183. (●) At constant pH, 4.5; (○) at variable pH; (▽) pH change during growth.

variable pH culture, a single activity zone at pH 4.0 was obtained in *Saccharomyces*, a single activity zone at pH 6.0 in *Kluyveromyces*, and three activity zones at acid, neutral and alkaline pH in *Rhodotorula* (Fig. 2).

As shown in Table 1, the three *Kluyveromyces* species had similar K_m values, but were from 3 to 7 times higher than those of *Saccharomyces* or *Rhodotorula*. *Kl. lactis* and *Kl. fragilis* were the most active, although they had different K_ms. The *Rhodotorula* strains were characterized by important activities at pH 7 and 12.

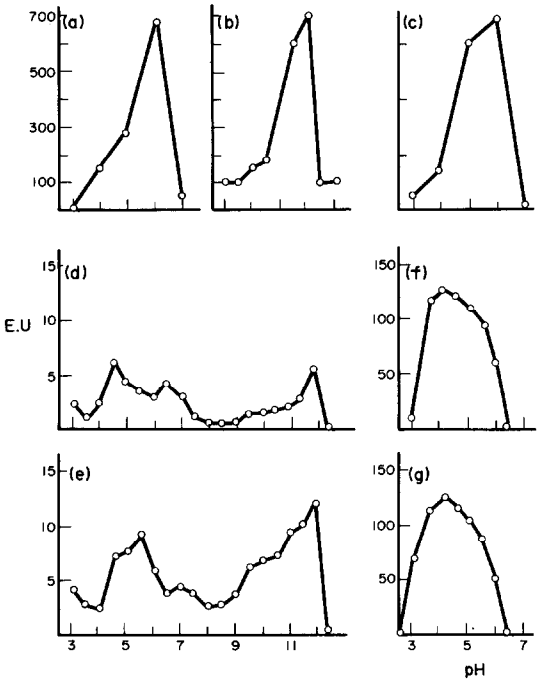

Fig. 2. Phosphatase activities of whole cells grown in D_0 medium at variable pH. (a) *Kl. fragilis*, (b) *Kl. lactis*, (c) *Kl. bulgaricus*, (d) *Rh. rubra* RMP, (e) *Rh. rubra* Rj1, (f) *S. cerevisiae* 4 and (g) *S. cerevisiae* 183.

After growing at a constant pH (4.5), only a single phosphatase activity was found for all the yeasts tested, always at pH 4 for *Saccharomyces*, pH 6 for *Kluyveromyces*, but at pH 6.5 instead of 7 for *Rhodotorula*. The K_m values were similar, but activities were decreased by 50 and 80%, respectively, in *Saccharomyces* and *Kluyveromyces*, and increased by 2–6 times in *Rhodotorula*.

Phosphatase activity of isolated cell walls

Isolated walls of *Saccharomyces* and *Kluyveromyces* at a variable pH exhibited a single phosphatase activity at pH 4 and 6, respectively. In the walls of *Rhodotorula*, the three acid, neutral and alkaline activities were again present (Table 2).

After growing at a constant pH (4.5), the same activities were found in the walls of *Saccharomyces* and *Kluyveromyces* but no activity was detected in the *Rhodotorula* cell walls.

Variation of phosphatase activities according to yeast treatment

Simple water washing decreased acid phosphatase activities only slightly in all strains. On the other hand, washing with sucrose solutions decreased this activity by 35%, *Kl. lactis* being the most sensitive to this treatment. This decrease was due to the liberation of enzymes by increasing osmotic pressure, not by ionic forces. Freeze-drying of cells also resulted in a decrease in the acid phosphatase activities.

In *Saccharomyces* and *Rhodotorula* this drop in activity, *ca* 70%, was due only to freeze-drying. In *Kluyveromyces*, on the other hand, freeze-drying caused a decrease in activity only if cells had been previously washed with sucrose and sodium chloride solutions, which rendered the enzymes soluble (Table 3). Cell disruption caused a drop of only 10–20% in the acid activities in all yeasts. However, for all disrupted cells, activities decreased markedly after washing in sucrose solutions. There should have been an increase in the enzymatic activities if they were linked to cell walls. In fact, the contrary was observed (Table 4).

Table 1. Characteristics of the phosphatases from the whole cell (culture at variable pH and at constant pH, 4.5)

	Strains										
	Saccharomyces cerevisiae		*Rhodotorula rubra*					*Kl. lactis*		*Kl. bulgaricus*	*Kluyveromyces Kl. fraglis*
	4	183	RMP			RJl					
(A) Culture at variable pH											
Optimum pH	4	4	3.5	7	12	5.5	7	12	6	6	6
K_m 10^{-4} M	4.5	3.5	3.6	8	27.7	2.5	10.4	166	12.5	27.5	20.0
E.U.	145	133	7.2	6.3	8.3	10	7.5	13	700	17	680
(B) Culture at a constant pH 4.5											
Optimum pH	4	4	3.5	6			6		5.5	4.5	—
K_m 10^{-4} M	4	3.7	0	5.5	0	0	2.7	0	16.5	28	—
E.U.	69.5	78	0	35	0	0	16	0	82.5	5.25	—

Activities were determined in 0.1 M sodium acetate buffer, except at pH 7: Tris–HCl, and at pH 12: glycine buffer. Yeasts were cultured in D_0 medium without phosphate.

0: No activity determined; —: activity not tested; E.U.: enzymatic units.

Table 2. Phosphatase activities of isolated cell walls of yeasts cultured at variable pH and at constant pH, 4.5

	Strains										
	Saccharomyces cerevisiae		*Rhodotorula rubra*						*Kluyveromyces*		
	4	183	RMP			RJ1			*Kl. lactis*	*Kl. bulgaricus*	*Kl. fragilis*
(A) Culture at variable pH											
Optimum pH	4	4	3.5	6.5	12	3.5	6.5	12	6	6	6
K_m 10^{-4} M	12	14.5	0.5	1.2	1.7	1.9	2.2	2.9	12.5	27.5	20
E.U.	5	8.9	6	12.5	26.3	3.3	13.5	29.5	360	3.4	200
(B) Culture at a constant pH of 4.5											
Optimum pH	4	4	3.5	6.5	12	3.5	6.5	12	6	6	6
K_m 10^{-4} M	26	27	0	0	0	0	0	0	16.5	25	—
E.U.	14	12.5	0	0	0	0	0	0	3.5	2.3	—

Activities were determined in 0.1 M sodium acetate buffer, except at pH 6.5: Tris–HCl, and at pH 12: glycine buffer. Yeasts were cultured in D_0 medium without phosphate.

0: No activity determined; —: activity not tested; E.U.: enzymatic units.

Table 3. Acid phosphatase activities of whole cells after different treatments

Strains	*S. cerevisiae* 4		*Rh. rubra* RMP		*Kl. lactis*		*Kl. bulgaricus*		*Kl. fragilis*	
Optimum pH	4		3.5		6		6		6	
Control: two washes in distilled water	145	100%	6.8	100%	700	100%	17	100%	680	100%
One wash in 60% sucrose	130	−10%	6.4	−5%	605	−13%	15.9	−6%	638	−6%
Five washes in 10% sucrose	115.5	−20%	5.4	−19.5%	520	−25.6%	13.8	−18.7%	554	−18.5%
Two washes in 1% NaCl	146.5	+1%	7	+3.7%	714	+2.5%	17.5	+2.5%	685	+0.8%
Yeasts washed in sucrose and NaCl*	108.5	−25%	5.3	−21.5%	460	−34.3%	14	−14.8%	629	−7.5%
Yeasts washed in distilled water and freeze-dried	43.5	−70%	2.2	−67.5%	599	−14.5%	13.5	−21.5%	632	−7%
Yeasts washed in glucose and NaCl* and freeze-dried	28.9	−80%	1.7	−74%	412.5	−41%	7.1	−58.3%	350	−48.5%

Activities expressed in E.U. (enzymatic units). % Activity (+ or −) test/control.

*One wash in 60% sucrose, five in 10% sucrose, one in 1% NaCl.

Table 4. Acid phosphatase activities after cell disruption

	Strains				
	S. cerevisiae 4	*Rh. rubra* RMP	*Kl. lactis*	*Kl. bulgaricus*	*Kl. fragilis*
Optimum pH	4	3.5	6	6	6
Control: two washes in distilled water	133	8.3	700	17	680
Washed and broken cells*	112	8.2	670	16.1	619.5
Broken cells washed twice in distilled water†	103	8	560	550	15
Broken cells washed twice in 1% NaCl†	109.5	8	495	12.1	475
Broken cells washed once in 60% sucrose†	72.6	5.1	485	12.6	473
Broken cells washed in 60% sucrose and freeze-dried	69.0	4.5	460	10.1	410

Activities: E.U./mg dry matter determined in 0.1 M sodium acetate buffer. Yeasts were cultured at variable pH, and were disrupted in an MSK Braun grinding mill for 9 min with glass beads.

*Activities determined on broken yeast cell suspension.

†Activities determined on centrifugation pellets after grinding and washing.

Acid phosphatase activity in culture media

Excretion of acid phosphatase in the media varied according to the strains and the conditions of the medium. *Saccharomyces* at variable pH excreted the greatest amount of phosphatase, *Rhodotorula* excreted most at constant pH (Table 5), while *Kluyveromyces* was little influenced by the culture conditions. It can be seen that in constant pH medium the amount of phosphatase excreted by *Rhodotorula rubra* corresponded to the neutral phosphatase activity, already stimulated in whole cells; tests for activities at pH 3.5 and 10 were negative, even on media concentrated 20 times by membrane ultrafiltration.

DISCUSSION

According to Lampen, invertase and acid phosphatase of *Saccharomyces* are covalently bound to wall components, since they are liberated by an enzymatic alteration of the cell walls [11, 12]. Kidby and Davies suggest that these enzymes are free but trapped between wall components, which explains their rapid liberation by sulphydryl derivatives able to break disulphide bonds [13]. More recently, Arnold mentioned a periplasmic location of these enzymes and excluded any covalent bond in the cell wall [9]. The culture conditions of micro-organisms influence the amount of enzymatic systems in walls [4, 14]. Thus glucose in the medium inhibits synthesis of cell wall and extracellular invertase [15], and similarly free orthophosphate ions in the medium inhibit acid phosphatase synthesis [2, 16–19]. Mizunaga distinguished between two phosphatases; one constitutive, being insensitive to orthophosphate ions, and another sensitive to these ions [17].

In previous work, Touimi Benjelloun and Bonaly observed that chloramphenicol and 2-hydroxybiphenyl in culture medium stimulated phosphatase activities in *Rhodotorula rubra*, where electron microscopic observation showed an alteration in the cell walls [20]. In the present work, all strains were grown under the same conditions; we observed in *Saccharomyces* and *Kluyveromyces* a single acid phosphatase activity, while in *Rhodotorula rubra* we confirmed the existence of three activities at the optimal pHs of 3.5, 7 and 11.

Phosphatase activities of isolated and purified cell walls were much lower. If these enzymes were linked to the wall, an increase in activity should have been observed, following an increased concentration of enzymes. In order to explain the observed decrease, whole yeasts were treated in the same way as purified walls. Washing whole cells and broken cells in sucrose solutions reduced activity to 35%, while washing in distilled water and 1% sodium chloride solutions had a very small inhibitory effect. Thus, enzymatic activities of yeasts were sensitive to the osmotic pressure but not to the ionic strength of the medium. Phosphatases were also altered by freeze-drying, as the activity decrease and K_m variations revealed. The above alteration was higher after washing the cells in sucrose and sodium chloride solutions.

The culture of yeasts at constant pH, which required the addition of sodium hydroxide and hydrochloric acid, increasing the ionic strength of the medium, decreased the phosphatase activities in all strains of *Saccharomyces* and *Kluyveromyces*. On the other hand, *Rhodotorula rubra* under these conditions excreted more phosphatases into the medium.

The excretion of proteins by *S. cerevisiae* depending on the pH of the medium was recently studied by Weller *et al.* [10]. An acid pH enhanced liberation while at neutrality excretion and even synthesis of proteins were inhibited.

In *Saccharomyces* and *Kluyveromyces*, variations of the phosphatase activities of cells and variations of the phosphatase excretions in the medium corroborated the findings of Weller *et al.* [10]. For *Rhodotorula rubra*, the culture at a constant pH of 4.5 enhanced excretion and did not inhibit the synthesis of phosphatase.

Finally, our results can hardly be explained by the hypothesis of covalent binding between phosphatases and cell wall components. They are in favour of a periplasmic location, following the hypothesis of Arnold [9], where they are fixed by links which can be easily ruptured.

EXPERIMENTAL

Micro-organisms. Seven yeasts belonging to three genera were used: *Rhodotorula rubra* strain RMP and a mutant, Rjl [21]; *Saccharomyces cerevisiae*, wild strain 4 and mutant strain 183 [22, 23]; *Kluyveromyces lactis*, *Kl. fragilis* and *Kl. bulgaricus*. The strains were maintained by subculturing at a 3-month interval on a beer wort medium and storing at 4°.

Culture media and methods. Culture medium D was prepared according to ref. [24]: glucose, 40 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; yeast extract, 1.5 g; 2 drops of anti-foam; H_2O, 1 l.; the pH was adjusted

Table 5. Acid phosphatase activities in culture media

	Strains						
	S. cerevisiae '4'	*S. cerevisiae* '183'	*Rh. rubra* RMP	*Rh. rubra* Rjl	*Kl. lactis*	*Kl. bulgaricus*	*Kl. fragilis*
Optimum pH	4	4	6	6	6	6	6
Variable pH medium							
E.U./l	34.4	24	?	2.2	3.5	1.5	2.3
K_m 10^{-4} M	5.8	4.7	—	1.7	5.9	13	6.1
Constant pH medium (4.5.)							
E.U./l	9.3	4.4	15.8	19	3.3	2.8	—
K_m 10^{-4} M	4.2	6.7	2.6	2.7	2.1	3.5	—

Enzymatic activities: E.U.: μM *p*-nitrophenol liberated in 1 ml of medium. Concentrated × 20 by membrane ultrafiltration (MW 10000). Activity was determined in 0.1 M sodium acetate buffer.

?: Very low activity; —: activity not determined.

to 5.6 before autoclaving for 20 min at 120°. Yeasts were cultured in 20 ml of the above medium in 100 ml conical flasks. Stirring was effected by magnetic bars rotating at 80 cycles/min. For the preparation of cell walls, yeasts were grown on a 20 l. Biolafitte fermentor aerated by a 'draft tube' system, with a 240 l./hr air flow. All cultures were maintained at 25°. Constant pH cultures were obtained by automatic addition of 0.1 M NaOH or HCl solns.

Growth curves. The growth of yeasts was studied by measuring *A* of the culture media at 620 nm and by weighing the dry extract.

Preparation of cell walls. After 18 hr growth in the fermentor, during the log phase, yeast cells were harvested by centrifugation, washed in H_2O, then crushed in an MSK Braun grinder with glass beads for 9 min. Walls were isolated and purified according to ref. [25]: washing in a 60% sucrose soln (once), 10% (5 ×), 1% NaCl (twice) and finally H_2O. Wall purity was controlled by phase contrast microscopy. Walls were then freeze-dried. All washings and centrifugations were done at 4°.

Determination of phosphatase activities (whole cells and walls). Method of ref. [26]. Na *p*-nitrophenylphosphate (Merck) was used as a substrate at a concn of 20 μM with a 2 ml reaction medium. 0.1 M NaOAc, 0.1 M Tris–HCl and 0.1 M glycine were used as buffers. The enzymatic reaction was effected at 30° for 30 min and stopped by the addition of 0.03 M NaOH and cooling at 0°. Liberated *p*-nitrophenol was measured by *A* at 405 nm. Results are expressed in enzymatic units (E.U.). One E.U. corresponds to 1 μM of *p*-nitrophenol liberated/min per mg dry wt.

Membrane ultrafiltration. Filtrations were effected on a Sartorius filter holder, with MW 10000 membranes, at 0° in an ice bath.

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